The tagging and capture hypothesis of synaptic plasticity: the roles of calmodulin kinases and the phenomenon of behavioural tagging.

Roger Lluis Redondo Pena

Ph.D Thesis

Submitted in satisfaction of the requirements for the degree of PhD in the University of Edinburgh, 2009.

Declaration

In accordance with postgraduate degree regulation of the University of Edinburgh, I declare that the work described in this document is my own, except where otherwise indicated, and that this thesis was composed by myself. The work here presented has not been submitted for any other degree or professional qualification.

Roger Redondo

Acknowledgements

Firstly, I would like to thank my supervisor Prof. Richard Morris for the opportunity to work with such a brilliant team in Edinburgh, for his guidance, patience and encouragement, and for always opening doors to fruitful collaborations. Among these, a special thanks to Prof. Haruhiko Bito and Dr. Hiroyuki Okuno for their insights into the molecular aspects of my research. Also, I am most grateful to Prof. Bruno Frenguelli, Dr. Tim Pearson, Dr. Rosalina Fonseca, Dr. Colin O'Carroll, and particularly to Mr. Patrick Spooner, for their technical expertise, which made the methodological advances in my research possible.

Dr. Emma Wood deserves special thanks as my thesis supervisor and for all her insight on the behavioural experiments in this thesis. Their success depended on the contributions of Hania Koever, Kathryn Berry, Zoe Richmond, Olivia Haggis, and specially Dr. Szu-Han Wang. Thanks to Dr. David Wyllie as the chair of the thesis committee and to doctors Bruno da Silva, Iona Beange, James Ainge, Iain Wilson, Stephen Martin, Rosamund Langston, Ingrid Bethus, Livia de Hoz, Stephanie Daumas, Tobias Bast, Marie Pezze, Kate Shires, Mark van Rossum, Cathy Vickers, Matthijs van der Meer and future doctors Dorothy Tse, David Bett, Cassie Hosburn, Noelia Madroñal, Steven Huang and to all my colleagues at the University of Edinburgh including the technical support offered by Charlie Marr, Richard Watson, Darren Downing, Anne Aitken and Jane Tulloch. Thanks to Jane Haley and all those involved in the promising neuroscience outreach programmes all around Edinburgh's schools.

Edinburgh and Scotland are made even more beautiful by the people that I have befriended here: To David and Iain Shaw, for keeping me in shape in football; to Tim, Deena and Kaia Mills and to all the proud Humanists in Scotland.

I want to express my gratitude to my family, natural and extended, for their encouragement and love. Thanks to my parents and brother for encouraging the pursuit of my dreams and giving me a great head start in life, the fruit of their hard-work and love. I dedicate my thesis to my best friend, my inspiring and unconditional supporter, my lovely wife Vanessa. May this work be one of the many fruits of our long and exciting path through a stimulating life together.

Abstract

The aims of this thesis were (1) to learn about the identities of the molecules involved in the maintenance of long-term potentiation (LTP), and (2) to develop and test a behavioural paradigm capable of elucidating the interaction between these molecular processes and the persistence of long-term memories.

By improving the stability of field recordings in *in vitro* electrophysiology, it was possible to investigate the molecular processes that determine the long-term changes in synaptic efficacy. In these experiments, the interactions between two convergent inputs onto the same neuronal population in the CA1 region of the hippocampus were monitored for over ten hours. Analytically powerful three-pathway protocols using sequential strong and weak tetanization in varying orders, and test stimulation over long periods of time after LTP-induction, enabled a pharmacological dissociation of potentially distinct roles of the calmodulin kinase (CaMK) pathways in LTP. This places constraints on the mechanisms by which synaptic potentiation, and possibly memories, become stabilized. The experiments show that tag setting is blocked by the CaMK inhibitor KN-93 that, at low concentration primarily blocks CaMKII, whereas a CaMKK inhibitor, STO-609, selectively limits the synthesis or the availability of plasticity related proteins (PRPs).

To test whether memories can be subject to modulation by independent experiences, behavioural studies tested the possibility of lengthening the persistence of a relatively weak memory by pairing its induction with an event capable of inducing the synthesis of the required PRPs. Corticosterone-dependent stressful events like a cold swim proved to interfere and weaken spatial memories. On the other hand, the exploration of a novel environment succeeded in rescuing the decay of a weak memory. The effect of the exploration of the novel environment was dependent on NMDA and dopamine receptor activation, as well as protein synthesis.

These results are discussed in relation to the synaptic tagging and capture hypothesis and a novel model of the neuronal mechanisms underlying synaptic plasticity is developed from them.

Abbreviations

5HT: Serotonin (5-hydrosytryptamine) Receptor; βA: Beta-adrenergic receptor; AA: Arachidonic Acid; AC: Adenylyl cyclase; AMPAR: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate receptor; AP5: (2*R*)-amino-5-phosphonopentanoate; bp-AP: back-propagating action potential; CA1, CA3: Cornus Ammonis area 1, area 3; CAM: Cell adhesion molecule, CaMK: calmodulin kinase; DAG: diacyl glycerol; ECM: Extracellular Matrix; E-LTP/D: early long-term potentiation / depression; fEPSP: field excitatory postsynaptic potentials (fEPSP); I3P: Inositol-3-phospate; LTP: long-term potentiation; LTD: long-term depression; L-LTP/D: late long-term potentiation / depression; mAChR: metabotropic AcetylCholinergic Receptor. MAPK: Mitogen-activated protein kinase; mGluR: metabotropic glutamate receptor; NMDAR: N-methyl-D-aspartic; PIP2: Phosphatidylinositol 4,5-biphosphate; PKA: Protein Kinase A; PKC: Protein Kinase C; PLA: Phospholipase A; PLC: Phospholipase C; PP1: Protein phosphatase 1; ProNP: Proneuropsin; PSD: postsynaptic density; PRP: Plasticity related protein; RyR: Ryanodine Receptor; STC: Synaptic Tagging and Capture; tPA: tissue plasminogen activator; VGCC: voltage gated calcium channels; VSCC: voltage sensitive calcium channel.

Chapter 1: Theoretical background

1.1 Introduction

In 1949, Donald Hebb advanced a hypothesis by which changes in synaptic efficacy could arise from the presynaptic cell's repeated and persistent stimulation of the postsynaptic cell (Hebb, 1949). Evidence supporting this hypothesis arrived with the recording of changes in field EPSPs in anaesthetized rabbits (Bliss and Lömo, 1973). This was the birth of long term potentiation (LTP), and the outset of the quest to learn if and how synaptic plasticity accounts for learned behaviour (Miles et al., 2005). Much progress has been made since, and in the 21st century the unknowns revolve around memory encoding, storage and consolidation.

Together with the ability to imagine the future, and to feel emotions, memories share a central role in sustaining human personality. The Scottish poet Alexander Smith said *'A man's real possession is his memory. In nothing else is he rich, in nothing else is he poor'* (Dreamthorp). In the ageing society of Western Europe, the increasingly common memory impairments associated with abnormal brain ageing reveal the sadness and emptiness of lives without memories. In health and disease, how do we remember and why do we forget? The ability and limits of human brains to maintain accurate memories and to determine what can later be recollected are fascinating. What determines then, whether a memory is encoded in such a way that it will allow the brain to recall it, by recognition or familiarity (Mandler, 1980)? Is it true that '*Things that were hard to bear are sweet to remember'* (Seneca)? In this 21st century, the century of the brain, we are in a privileged position to work out the intricate mechanisms of memory formation. The reductionist approach behind the current momentum in brain research allows for controlled chemical manipulation of the putative molecular processes responsible for memory formation and retrieval. As another drop into the glass of neuroscientific knowledge, my research aims to discover some details about the electrophysiology of synaptic changes at the same time that I connect this knowledge to the macroscopic behavioural world of memories.

In the work that constitutes this thesis, I have used molecular and electrophysiological tools to look into what is necessary for neurons in the hippocampus to sustain changes in the efficacy of their connections. Also, I have translated our knowledge about the cellular mechanisms of plasticity into devising novel behavioural protocols to study memory interactions. The aims of this thesis were (1) to develop and use a method to dissociate the role of particular molecules in the local and cell-wide processes leading to synaptic plasticity, with particular reference to the role of calcium-calmodulin kinases (CaM Kinases) in the local setting of a synaptic tag or in the production of diffusible plasticity related proteins (PRPs), and (2) to translate the electrophysiology of heterosynaptic plasticity into a behavioural correlate based on spatial memory.

1.2 The classical properties of synaptic plasticity in the hippocampus

Animal behaviour is the end result of activity in the nervous system. Learning new behaviours or the modification of existing ones must require a change somewhere in that nervous system. What changes take place, where and how do they occur? During early body and brain development, pre-programmed patterns of growth and connectivity are laid out following chemical blueprints mostly hard-wired in our genetic makeup. At some point during development, some nervous systems display their susceptibility to influences by the environment in which they grow. What is the difference between a brain capable of performing a particular behaviour and that same brain minutes or days before learning took place? Neuroscientists now agree that most if not all learning is ultimately based on changes at the connections between neurons (synapses). This plasticity allows new patterns of activation between the neurons involved in sensing stimuli and those in charge of motor or hormonal responses. Synaptic plasticity is required for a brain to change its response to a given stimulus, but this plasticity follows certain rules or properties that have been identified.

The favoured Hebbian model of synaptic plasticity (Hebb, 1949) has the following properties:

- Use-dependency: plastic synapses are found on cells that are activated as part of the encoding of memory.
- Associativity: synapses that are active and contribute to the activation of the cell via its depolarization will show plasticity (Gustafsson et al., 1987)
- Specificity: Synaptic change is selective requiring that inactive synapses in the vicinity of those active are unaffected (Bliss and Lömo, 1973).

Cellular biology puts some constraints as to what cellular processes can account for the three properties of Hebbian synaptic plasticity. One of the challenges is the molecular turnover experienced by the synapses undergoing plasticity changes (Ehlers, 2003). The source of new molecules is the genetic material stored in the nucleus of the cell. The DNA that encodes for the proteins necessary to allow the

plastic changes in synapses is transcribed into mRNA far from where those changes will ultimately take place. At any one time, only a small subset of all the synapses will require the products of these genes. How do the gene products find their way to the appropriate synapses? Or how do the appropriate synapses know when and what gene products they require? This is certainly a problem for molecular biologists and electrophysiologists but as synaptic plasticity is theorized to be the basis of learning and memory, it has implications for neuronal models of brain function as well as for the clinical understanding of diseases of the mind.

1.3 Electrophysiology of synaptic plasticity

Synapses between neurons can change in strength such that the probability of a response in the postsynaptic neuron to the same input from the presynaptic neuron is altered (i.e. synaptic strength or efficacy). A decrease in strength is referred to as a depression, while an increase is called a potentiation. Thus, this form of synaptic plasticity is often referred to as long-term depression (LTD) and long-term potentiation (LTP).

One can measure synaptic strength in a neuronal circuit by looking at field excitatory postsynaptic potentials (fEPSP). A fEPSP is the recording of the movement of ions due mainly to glutamatergic receptor opening and offers a direct measurement of the strength of the synaptic response (Kandel and Spencer, 1968). Field EPSPs can be measured relatively easily from the *stratum radiatum* of the CA1 region of the hippocampus (anatomy described in section 1.7.2). The shape of the fEPSP allows for measurements of its slope and amplitude and these are direct correlates of the voltage changes and the synaptic response around the recording electrode. Most of the electrical potential that is recorded is due to the movement of sodium and potassium ions, with some minor chloride and calcium components too. The ionic movements responsible for a fEPSP are due to the opening of postsynaptic receptors after the release of the excitatory neurotransmitter glutamate. The two ionic glutamate receptors are the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) and the N-methyl D-aspartate (NMDAR). AMPARs react and open faster than NMDAR and consequently, if one blocks AMPARs the field EPSP loses its initial slope while keeping much of its late amplitude. Blocking NMDAR keeps the initial slope constant but decreases the late amplitude (Xiao et al., 1995). Under stable conditions, the application of pulses of electrical current of constant intensity at very low frequency (i.e. e. 0.0067 Hz) recruits axonal fibres and elicits fEPSPs that can be recorded for hours at a constant amplitude and slope (i.e. test stimulation fEPSP). However, if at some point, specific patterns of high frequency stimulation are applied, the fEPSP elicited after renewed test stimulation changes by increasing both its slope and amplitude (Bliss and Lömo, 1973). When this change in the synaptic response lasts more than one hour it is called long-term

potentiation (LTP) (Kelleher et al., 2004b). Other patterns of stimulation may reduce the synaptic strength (i.e. long-term depression (LTD)). These changes are the basis of the synaptic plasticity and memory hypothesis whereby neuronal plasticity accounts for memory storage (see below).

1.3.1 Induction of long-term potentiation

Activation of NMDAR by specific patterns of pre- and postsynaptic activity triggers calcium-dependent second-messenger systems that start the process of induction of LTP. Calcium influx is a critical aspect of LTP, but not for synaptic transmission, paired pulse facilitation (PPF) or short-term potentiation (STP) (Dunwiddie and Lynch, 1979). Calcium can act presynaptically, increasing quantal content (the number of vesicles released in response to a nerve impulse), or postsynaptically, increasing quantal size (the synaptic response to a single vesicle). Indeed, LTP induction increases the probability of vesicle content release in presynaptic terminals (Zakharenko et al., 2001). However, because *postsynaptic* calcium influx is necessary to elicit LTP, whatever changes take place presynaptically, they must depend on a retrograde messenger. Calcium is thought to play a crucial role in the induction of plasticity and it determines which mechanisms of expression will be engaged (i.e. modest increases in calcium may lead to LTD whereas larger increases lead to LTP (Yang et al., 1999)). This picture may be over simplistic since the timing and the source of the calcium may be critical in determining the direction of the plastic change (LTP or LTD). As explained in the next section, spike-timing dependent plasticity reveals that more presynaptic pulses, with the consequent increase in calcium influx, do not necessarily facilitate the induction of LTP (Wang et al., 2005).

In any case, the sources of calcium are under investigation. Calcium enters the postsynaptic dendrite through NMDAR once the voltage-dependent Mg^{2+} block has been removed. Membrane depolarization induces a conformational change in the NMDAR, releasing the Mg^{2+} ion and allowing calcium influx. Ca^{2+} can also enter the postsynaptic cell after metabotropic glutamate receptors (mGLUR) activation, which through second messenger cascades, release Ca^{2+} from internal stores. This is known as Ca^{2+} induced Ca^{2+} release (CICR). Other sources of Ca^{2+} are influx through

GluR2-containing AMPARs (Kim et al., 2001; Guire et al., 2008) and through the opening of voltage dependent calcium channels (VDCC). However, synaptic plasticity is not determined only by how much calcium is released but also by the timing and the origin of the calcium rise.

The role of back-propagating action potentials and dendritic spikes

Ca^{2+} can be released into the postsynaptic spine independently of presynaptic activity via back-propagating action potentials (bp-AP). Pharmacological analysis has shown that CA1 spines contain mostly R-type VSCCs (Sabatini et al., 2001) and that LTP induction is inhibited by $Ni(^{2+})$, an R and T-type VDCC blocker. This reveals a contribution of these channels to Ca^{2+} influx (Isomura et al., 2002). The additional Ca^{2+} influx driven by bp-AP is capable of engaging the synthesis of plasticity related proteins and therefore contribute to the maintenance of LTP (Raymond, 2008). Whether or not the actions of a bpAP are necessary for the induction of LTP is still a matter under discussion since isolated dendrites successfully show LTP (Frey et al., 1989; Vickers and Wyllie, 2007), blockage of bp-action potentials with TTX still allows for LTP (Remy and Spruston, 2007) and bp-AP are not enough to activate CREB in hippocampal neurons of young animals (Deisseroth et al., 1996). The age of the animal is another factor to take into account since only adult CA1 pyramidal cells seem to rely on somatic spikes to induce plasticity (Buchanan and Mellor, 2007).

1.3.2 Expression of synaptic plasticity

The processes of induction lead to the phenomenon of expression of LTP, whereby a change in synaptic strength is measured for more than 30 minutes. Here, I focus on the potentiation (LTP) of the synaptic efficacy, with depression (LTD) discussed later. What accounts for this change in synaptic efficacy is open to discussion.

There are presynaptic modifications (more glutamate released) (Sokolov et al., 2002), postsynaptic modifications (changes in neurotransmitter receptors), extrasynaptic changes (less uptake of glutamate), and morphological modifications (spine growth) (Bliss and Collingridge, 1993). The presynaptic changes seem to be downstream from the major role of postsynaptic calcium entry through NMDARs

and the consequent postsynaptic increase in the AMPAR response to synaptic stimulation (Davies et al., 1989). However, even though it is the postsynaptic calcium entry that triggers the mechanisms of expression, the late expression seems to be driven primarily by presynaptic changes in the probability of release of neurotransmitter (Bayazitov et al., 2007; Enoki et al., 2009).

We know, however, that the induction of LTP activates multiple signalling cascades (Lynch, 2004). Stimulation by release of glutamate enlarges spines and this is associated with an increase in AMPAR-mediated currents at the stimulated synapse and is dependent on NMDA receptors, calmodulin, CaMKII and actin polymerisation (Matsuzaki et al., 2004). After induction of LTP there is incorporation of glutamate receptors (AMPAR and NMDAR). Interestingly, those same receptors are removed after LTD induction (Heynen et al., 2000; Andrasfalvy and Magee, 2004). The complexities of AMPAR regulation during LTP are reviewed by Song (Song and Huganir, 2002).

Lisman proposes a model for the expression of LTP that introduces the concept of hyper slots in a synapse, which consist of smaller slots to be filled by AMPARs (Lisman and Raghavachari, 2006). Immediately after LTP induction, more hyper slots are added to the synapse and more AMPARs are added to the slots. Also more glutamate is released from the same number of vesicles as now instead of a 'kiss and run' mode of fusion, they deliver neurotransmitter by 'full fusion' mode (presynaptic change). These three things account for the increase in quantal size and quantal content. After 30 min or 1 h, GluR1 levels within slots fall, bringing the quantal size to pre-LTP levels. However, the hyper slots (i.e. the AMPA modules) and the increased vesicle release remain, contributing to the sustained increase in quantal content (Sokolov et al., 2002).

Presynaptic changes

There is a presynaptic component to LTP in the form of a sustained increase in glutamate release (and not a decrease in glutamate re-uptake from the synaptic cleft) that correlates with the increase in synaptic vesicle proteins (synapsin, synaptotagmin and synaptophysin) 3 hours but not 45 minutes after LTP in the Dentate Gyrus

(Lynch et al., 1994). The presynaptic increase in synaptophysin colocalizes with postsynaptic increases in GluR1 in CA1 synapses (Antonova et al., 2001).

The ways to increase neurotransmission (LTP) from the presynaptic side include an increase in active zones, an increase in Ca^{2+} channel numbers and proximity to vesicles, larger vesicles as well as an increase in their docking and priming for fusion (Atwood and Karunanithi, 2002). Exocytosis involves Sec18/N-ethylmaleimide-sensitive fusion protein (NSF), Sec17/soluble NSF attachment proteins (SNAPs), SNAP receptors (SNAREs), Sec1/Munc18 homologues (SM proteins) and small GTPases of the Rab family (Rizo and Sudhof, 2002). During exocytosis, syntaxin attaches to the membrane and SNAP25 to the vesicle through Synaptobrevin. SNAP25 threads into Syntaxin and brings the vesicle in close contact with the cell membrane. Ca^{2+} facilitates the fusion of the vesicle and after that NSF and SNAPs disassemble the SNAP25-syntaxyn complex.

Role of dendritic spines in the expression of plasticity.

Anatomical studies carried out in conjunction with induction of long-term potentiation (LTP) in the dentate gyrus of the hippocampus (Desmond and Levy, 1986a) indicated that excitatory stimulation leads to changes in spine morphology and increase in total surface area of postsynaptic density (Desmond and Levy, 1986b). Others have reported a major increase in synapse number (Buchs and Muller, 1996) and synaptogenesis (Maletic-Savatic et al., 1999) in response to stimulation of the CA1 region of the hippocampus. Conformational changes in dendritic spines depend on the actions of actin (Fischer et al., 1998) while tetanic stimulation induces the targeting of actin regulatory molecules to the spine head (Ackermann and Matus, 2003). What is the role of dendritic spines in the expression of synaptic plasticity?

Dendritic spines are tiny membranous compartments consisting of a head (volume ~0.01-1 μm3) connected to the parent dendrite by a thin (diameter ~0.1 μm) spine neck (reviewed in (Harris, 1999)). Each spine contains a postsynaptic density (reviewed in (Kennedy, 2000)), some have smooth endoplasmic reticulum (SER), while others are filled with polyribosomes. The spine neck may serve to restrict diffusional exchange of signalling molecules between the spine head and parent

dendrite or to impede synaptic currents. The spine neck acts as a diffusion barrier (100 times slower than free diffusion). On the other hand, spine neck electrical resistances estimated from measured diffusional resistances have confirmed that spines are not capable of regulating synaptic strength through changes in their resistances (Svoboda et al., 1996; Majewska et al., 2000).

Uncaging of glutamate elicits LTP in a very localized manner in which surrounding dendrites remain unaffected (Matsuzaki et al., 2004). This single spine LTP is NMDA, CaMKII and actin polymerisation dependent. More importantly to this thesis, these spine changes are restricted locally and may have a specific role to play in the modulation of heterosynaptic plasticity as described by the synaptic tagging and capture hypothesis (see below). Concerning the role of spine morphology in LTP maintenance, the question remains as to whether morphological spine change is required for synaptic plasticity. Even though cell cultures show spine changes after glutamate uncaging that depend on NMDA, calmodulin, CaMKII activation and actin rearrangement (Matsuzaki 2004), there are studies in acute brain slices that show synaptic plasticity with only transient conformational change in the spines of the target neurons (Lang et al., 2004). Whatever the molecular actions and reconfiguration responsible for the expression of changes in synaptic efficacy, the molecular turnover experienced by the synapse makes it necessary to explain how these changes are maintained.

1.3.3 Maintenance of synaptic plasticity

Whatever cell signalling cascades are engaged after LTP induction, the question remains as to what keeps the change stable under the molecular turnover present in all biological systems, including membrane complexes like the synapse. Some biochemical changes will account for the induction or the expression of LTP, but others will be responsible for making this expression stable.

NMDAR-dependent LTP requires the activation of various kinases as well as the synthesis of plasticity-related proteins (PRPs) at different time points after induction. The need for distinct molecules at different time-points allows us to divide synaptic potentiation into phases (Bliss and Collingridge, 1993) (see below). With the activation of kinases, phosphorylation of their substrates and modifications in

receptor number and properties being conditions for LTP expression, the synthesis of new molecules appears to be an additional factor in LTP maintenance (Krug et al., 1984; Stanton and Sarvey, 1984). A goal is to find out the identity of these proteins, the pathways that lead to their synthesis and what happens at synapses that use them (Abraham and Williams, 2003).

We know that LTP leads to the phosphorylation of the cAMP response element-binding protein (CREB) which then binds to the cAMP-dependent response element (CRE) in the promoter regions of certain genes (Impey et al., 1996). There are multiple biochemical pathways capable of engaging this gene activation (Shaywitz and Greenberg, 1999) and a two of them (CaMKK and PKA) will be the subject of investigation in this thesis.

Concerning the identity of plasticity related genes, several studies point to transcription factors, cell signalling, structural molecules and enzymes that are upregulated after high frequency stimulation (Abraham and Williams, 2003). An extended list with particular roles for each of them will be described in the section concerning the plasticity related molecules necessary for the maintenance of LTP.

There is a clinical aspect of this basic research on the mechanisms of LTP. There is evidence for deficits in memory correlating with impairments in the expression or the maintenance of LTP (Bliss and Collingridge, 1993; Barad et al., 1998; Bach et al., 1999; Malleret et al., 2001; Scharf et al., 2002; Hill et al., 2004; Lynch, 2004; Kim et al., 2005; Nagy et al., 2006; Tamura et al., 2006a; Chan et al., 2007; Nie et al., 2007; Smolen, 2007; Zhou et al., 2007). Assuming a tight correlation between the mechanisms behind synaptic plasticity and those responsible for the establishment and maintenance of memories, learning about the maintenance of synaptic plasticity should inform us about the requirements for memory persistence. Already this knowledge has allowed better treatment of conditions like post-traumatic stress disorder (PTSD) by using a compound known as fundamental for the maintenance of long-term plasticity (i.e. beta-adrenergic blockade of emotional memory (Reist et al., 2001)).

Synthesis of plasticity related proteins (PRPs)

The molecular machinery necessary for the translation of mRNA into proteins has been found in the dendrites of neurons, making possible a role for local protein synthesis (Steward and Fass, 1983). There is a wide variety of mRNAs found in the dendritic compartment and this variability is found both within and across different cell types (Steward, 1997). There are cytoplasmic, cytoskeletal, integral-membrane, and membrane-associated proteins.

Is dendritic protein synthesis necessary for the maintenance of synaptic plasticity? Dendritic mRNA, coding for the kinase CaMKII, is translated after the induction of synaptic plasticity (Ouyang et al., 1997) but whether this translation is necessary for the maintenance of LTP is still under investigation (Steward and Schuman, 2001). Severing the dendrites from the soma is one way of addressing this as protein synthesis in isolated dendrites can still occur (Tsokas et al., 2005). In isolated dendrites L-LTP can be maintained and is sensitive to translation but not to transcription inhibitors (Vickers et al., 2005). A previous study did show the opposite results, suggesting somatic protein synthesis is important (Frey et al., 1989).

The localization of mRNA for plasticity proteins in the dendrites does not resolve the problem of targeting gene products to the correct sites at synapses. This problem is also faced by mRNA targeting to synapses. Evidence for the targeting of mRNAs exists in the targeting of the IEG Arc to activated sites on dendrites after synaptic activation (Steward et al., 1998). Studying the targeting of mRNAs can reveal which transport molecules are involved and by which mechanisms (i.e. what targets capture them on arrival at the correct synapse). For mRNAs, the molecule staufen, originally found in *Drosophila* but with related genes in mammalian hippocampal neurons (Wickham et al., 1999), moves together with RNA along microtubules in dendrites. What directs staufen to a particular synapse is unknown.

1.3.4 Long-term potentiation and depression?

Synaptic efficacy can be strengthened (LTP) as well as weakened (LTD). What determines the direction of this change? Is a difference in the induction and expression mechanisms involved, or does the maintenance of LTP also have different properties than that of LTD?

One possibility is that the level of cytoplasmic calcium determines the direction of the change in synaptic plasticity. NMDAR, IP3 and VGCC act together to raise cytoplasmic [Ca^{2+}]. Low increases in calcium activate calcium calmodulin (Ca/Calmodulin) that in turn activates calcineurin (PP2B) that inactivates inhibitor 1. Inhibitor 1 keeps protein phosphatase 1 (PP1) inactive, but fails to do so after the action of PP2B. PP1 then dephosphorylates AMPAR and CaMKII (Kemp and Bashir, 2001). Raised calcium levels can therefore elicit LTD. Additionally, and as explained when discussing the induction of LTP, it is also calcium influx that induces the potentiation of the synaptic response. The detector of how much calcium enters the cytoplasm is Ca/Calmodulin. With low increases of calcium, Ca/Calmodulin activates PP1 while with high calcium Ca/calmodulin activates adenylyl cyclase (AC) and protein kinase A (PKA) that phosphorylates inhibitor 1 and that stops PP1 (Lisman, 1989). To summarize Lisman's model: Low increases in [Ca^{2+}] activate PP1 and do not allow the autophosphorylation of CaMKII while high levels autophosphorylate CaMKII and inhibit PP1 through inhibitor 1 and AC. Interestingly, the AC to PKA pathway that activates inhibitor 1 could also be independently activated by dopaminergic input.

To summarize, different outcomes in the expression of synaptic plasticity (potentiation or depression) could depend on what receptors are activated (Liu et al., 2004), on how much calcium is released into the cytoplasm (Lisman, 1989; Yang et al., 1999; Ismailov et al., 2004), or on the timing and source of the calcium increase (Wang et al., 2005).

1.3.5 Depotentiation is different from depression

The history of the synapse is decisive in determining what mechanism will produce a decrease in synaptic efficacy: LTD induction in naïve synapses dephosphorylates the PKA site of AMPARs, whereas in potentiated synapses it dephosphorylates the CaMKII site. Conversely, LTP induction in naïve synapses phosphorylates the CaMKII site, whereas in depressed synapses LTP phosphorylates induction the PKA site (Lee et al., 2000).

Depotentiation (DP) involves the reduction of synaptic strength in synapses that have previously been potentiated, while LTD is a reduction in the synaptic strength of naïve synapses. Depotentiation seems to be a different process than LTD since one involves the phosphorylation of Ser-831 of the GluR1 subunit of the AMPAR (LTP; depotentiation) and the other that of Ser-845 (LTD; de-depression) (Lee et al., 2000; Kemp and Bashir, 2001). Depression (LTD) is observed easily in the hippocampal slice (Stanton and Sejnowski, 1989; Christofi et al., 1993), while it is more difficult to elicit *in vivo* unless one works with young animals (Errington et al., 1995), suggesting that LTD may be restricted to the immature, developing hippocampus. In other brain structures like the cerebellum, LTD may be the main mechanism of synaptic plasticity and has already been linked to motor memory (Aiba et al., 1994).

The mechanisms for LTD and LTP discussed in this chapter will integrate the results of this thesis and be updated in a proposed model described in chapter 11.

1.4 The synaptic tagging and capture (STC) hypothesis of synaptic plasticity.

1.4.1 The STC hypothesis

NMDAR-dependent long term potentiation (LTP) of the synaptic response to electrical stimulation in the CA1 region of hippocampal slices can be divided into an early-phase (protein-kinase dependent) and a late-phase (protein-synthesis dependent) (Matthies, 1989b). Weak tetanization produces a potentiation that decays by approximately three hours called early-LTP (E-LTP). Strong tetanization, in contrast, elicits a potentiation that lasts for at least six hours (late-LTP (L-LTP)). This L-LTP requires a critical period of transcription around the time of induction as protein synthesis blockers during induction prevent the maintenance of LTP (Krug et al., 1984).

The stimulation of two independent sets of axons convergent onto the same neuronal population in CA1 allowed for an interesting discovery. The block of L-LTP by the protein translation blocker Anisomycin after the strong tetanization of one pathway could be rescued if, before the application of the drug, the other set of synapses had experienced the strong high-frequency stimulation. In this case, both sets of synapses were capable of showing L-LTP (Frey and Morris, 1997).

Another interesting observation was made when the two strengths of stimulation (weak and strong) were given within 60 minutes of each other to two independent pathways that project to the same neuronal population. Now, the weak tetanus is capable of producing L-LTP (Frey and Morris, 1997).

To account for these results, the synaptic tagging and capture (STC) hypothesis was proposed. This identifies two dissociable events during synaptic plasticity. The first event involves the local setting of a synaptic tag which sequesters the gene products synthesized and distributed diffusely by the second event, protein-synthesis (PS). In the two-pathway experiments, plasticity-related-proteins (PRPs) become available and are sequestered at the weakly stimulated synapses, or at the synapses tetanized in the presence of anisomycin, allowing LTP to be maintained longer than three hours.

The STC hypothesis and the experiments that it manages to explain reveal a fundamental piece of information concerning the mechanism of synaptic plasticity. The heterosynaptic effect seen in experiments involving the tetanization of inputs from two independent but convergent pathways reveal a novel set of events necessary for the maintenance of synaptic plasticity. One is a local change at or around the synapses that have changed their synaptic weights (setting of a tag). Another is the synthesis of PRPs that are diffusible and mobile. The third is an interaction between the local tag and the PRPs such as to allow local changes in synaptic efficacy to be consolidated. The block of any of these three steps impairs the maintenance of long-term changes in synaptic strength.

At the outset, little was known about the molecules involved in the setting of the tag or in the synthesis of PRPs (Martin and Kosik, 2002). Part of the work of this thesis aims to discern which molecules are necessary for synaptic tagging and capture. A novel testing methodology was developed to assess the role of particular molecules in the cascade of events leading to either the setting of the tags or the synthesis of PRPs.

1.4.2 Cross-tagging

One of the most interesting questions concerning the STC hypothesis is how the properties and elements described in it (tag, PRPs, their interactions,) interact between potentiation and depression. Long-term depression (LTD) of the synaptic response in the Schaffer collateral input into the CA1 pyramidal cells follows many of the properties of LTP, including the rescue of E-LTD into L-LTP in one input when there is a prior induction of L-LTD in another convergent pathway (Kauderer and Kandel, 2000). The immediate follow up questions after the finding of synaptic tagging and capture in LTD are: how much does STC-LTD resemble STC-LTP, and do tags and PRPs in LTD and LTP interact?

Sajikumar and Frey (Sajikumar and Frey, 2004a) confirm STC-LTD and they also show that there is cross-tagging between LTP and LTD. Firstly, the tag in STC-LTD follows a very similar time course (between 1 and 2 h) as that of STC-LTP (Frey and Morris, 1998b). Secondly, ‘weak before strong’ protocols work as well as ‘strong before weak’ protocols in showing the rescue of E-LTP into L-LTP. Thirdly,

D1/D5R antagonists specifically block the synthesis and availability of PRPs while allowing functional tags to capture PRPs provided by a pathway not affected by the drug. But most importantly, the PRPs required for the rescue of early phases into late phases can be brought about both by a strong LTP inducing stimulus as well as by a strong LTD inducing protocol. Which strong induction method is used makes no difference to the rescue of E-LTP or E-LTD into their longer lasting late phases. There are several mutually exclusive interpretations of this finding:

1 LTP uses different tags than LTD but they share the same PRPs for their maintenance.

2 LTP uses different tags than LTD as well as different PRPs but the cell produces both types of PRPs once stimulation reaches the threshold for L-LTP or L-LTD induction. This is supported by the discovery of the role of PKMζ (see below).

3 Tags and PRPs are the same because the mechanisms of expression of a change in synaptic response are independent of the molecular switch necessary for the maintenance of those changes (tag) and the PRPs that the switch requires to stabilize changes in synaptic strength (see the resetting of the tag below).

1.4.3 Resetting the tag

If our understanding of the tag points to a highly relevant role for protein phosphorylation as its main mechanism of action, then the dephosphorylation of the right target would predict the elimination of the tag. The first clues of this possible switch property of the tag became apparent when mGluR activation was shown to alter the state of a synapse in such a way that subsequent LTP induction would not require mGLuR activation again. This switch acts locally in a synapse-specific way and depends on protein kinase activation (Bortolotto et al., 1994). Following our understanding of depotentiation and the effects of low but prolonged rises in Ca^{2+} concentration, low-frequency stimulation applied after mGluR activation was capable of turning off the switch.

Subsequent research pinpointed CaMKII as a possible kinase involved in the switch property of the tag. The CaMKII inhibitor KN-62 could prevent the setting of the molecular switch at a concentration in brain slice experiments that was

subthreshold for inhibiting the induction of LTP (Bortolotto and Collingridge, 1998). These findings reveal a dissociation between the expression of a change in synaptic plasticity (LTP) and the setting of the tag (more on this in chapter 6). Low frequency stimulation resets the molecular switch by specifically blocking the tag since it still allows the PRPs to be made available heterosynaptically (Young and Nguyen, 2005). In a series of technically elegant experiments, Young et al., 2005 show how 5 Hz stimulation for 3 min is capable of preventing L-LTP in a strongly tetanized set of synapses while another set of independent but convergent synapses succeeds in maintaining L-LTP even if it is only weakly tetanized. This type of experiment allows for the dissociation between elements necessary for the setting of tags and those necessary for the synthesis of PRPs and are the inspiration for the electrophysiological paradigms used in my research (described below). The timing of the LFS is critical since it appears that 10 min after tag-setting, the tags are immune to the effect of LFS (Sajikumar and Frey, 2004b).

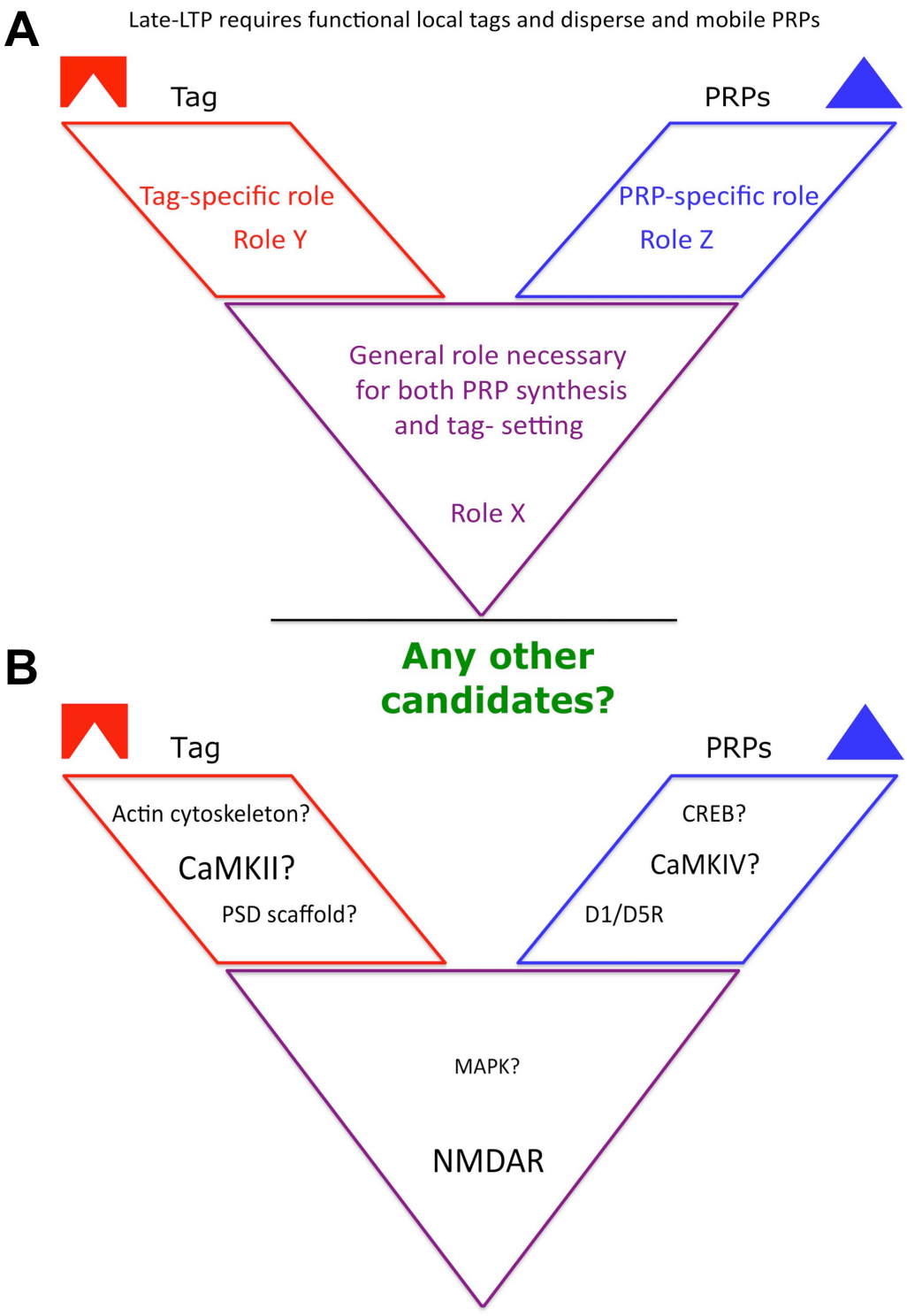
1.4.4 Assigning roles to molecules

Theoretically, within the complex set of chemical interactions behind LTP there are molecules that are necessary for the setting of the tag but not necessary for the synthesis of PRPs (Tag role). In a complementary way, there may be molecules with actions necessary for the synthesis and availability of PRPs but without a necessary role in the setting of the tag (PRP role) (Figure 1.1). Due to the multiple interactions among molecular pathways, the number of event-specific molecules will depend on the level of fine-tuning and on the redundancy of enzymatic cascades involved in synaptic plasticity. In any case, irrespective of how many molecules have a generalist role and are necessary for both the setting of the tag and the synthesis of PRPs, it is possible to imagine a particular molecule whose role is necessary for the setting of the tag but not for the signalling that leads to the synthesis of PRPs (Y role in Fig 1.1). One can also hypothesize candidate molecules whose role is only required to make plasticity proteins available while not having an indispensable role in the setting of the synaptic tag (Z role in Fig 1.1).

Figure 1.1 Potential roles for different molecules in the pathways leading to PRP availability.

A. Theoretically, one can predict that a molecule Involved in the maintenance of LTP could be necessary both for the setting of tags and the synthesis and availability of PRPs (role X). Another hypothetical molecule could play a necessary role in tag setting while not being necessary for PRP availability (role Y) and vice versa (role Z).

B. Based on our current knowledge of synaptic plasticity in the CA1 region of the hippocampus, where STC has been reported, we can assign certain roles to some molecules. We know that NMDARs are necessary for both the setting of the tag as well as the synthesis of PRPs (role X, at the stem of the diagram) (O'Carroll and Morris, 2004). Dopaminergic action through D1/D5Rs, on the other hand, is only necessary for the availability of PRPs (role Z) (Sajikumar and Frey, 2004a). What are the roles of other molecules implicated in LTP?


A
Late-LTP requires functional local tags and disperse and mobile PRPs
Tag
PRPs
Tag-specific role
Role Y
PRP-specific role
Role Z
General role necessary
for both PRP synthesis
and tag- setting
Role X
B
Any other
candidates?
Tag
PRPs
Actin cytoskeleton?
CaMKII?
PSD scaffold?
CREB?
CaMKIV?
D1/D5R
MAPK?
NMDAR

1.4.5 Two steps towards role identification

To distinguish between the X, Y and Z roles (Figure 1.1, see figure legend for details) we can devise two types of experiments. All are based on the appropriate stimulation of two independent pathways onto the same population of neurons (CA1 pyramidal cells). Their particularities stem from which strength of stimulation is used on each pathway and when drugs blocking the effect of the candidate molecules are present in the preparation.

Test for 'PRP block' (Strong before strong, Figure 1.3)

First, an inhibitor of the candidate tag molecule has to be capable of blocking L-LTP if present at the time of induction with strong tetanization. If the inhibitor can be washed out of the preparation, the strong tetanus can be delivered to one pathway under its influence, then washed out, and later another strong tetanus can be applied to the second pathway (i.e. strong-with-drug-before-strong). The synapses of the second pathway should have no problem expressing and maintaining L-LTP. This is because after the drug washed out, the stimulation that they receive would be capable of both setting local tags and engaging the synthesis of mobile PRPs. The critical variable in this type of experiments is what happens to the synapses that are strongly tetanized under the influence of the inhibitor (i.e. the first pathway). If they succeed in maintaining L-LTP, we have to assume that the synapses were capable of making use of PRPs (from the second stimulation). This means that even under the effect of the inhibitor capable of blocking L-LTP in control experiments, the setting of local tags remains functional so as to allow capture once the PRPs are brought about by a second stimulation. In this case, the inhibitor must be blocking molecules necessary for the synthesis and availability of PRPs, but with no necessary role on the sequence of events leading to the setting of local tags. This outcome has been reported both with anisomycin experiments and with dopamine blockers too (Frey and Morris, 1997; Sajikumar and Frey, 2004a).

The alternative outcome of the strong-before-strong experiment is that the pathway stimulated under the influence of the drug of interest fails to show L-LTP. In this

case, we know that the PRPs were available since the second pathway was capable of using them to maintain its potentiated state. We conclude then that the inhibitor prevents the setting or action of the tag at the synapses where it acted. However, this outcome is less selective analytically as it does not clarify whether the block of the tag is specific or whether the inhibitor might also block the synthesis of PRPs. This type of result has been reported before (Frey and Morris, 1997; Sajikumar et al., 2007) but needs a further set of experiments to clarify the action of the inhibitor (described below).

If the inhibitor cannot be washed out but exerts its effect exclusively during the induction of LTP and not during its maintenance, the protocol can be altered in such a way that the strong stimulation without the drug is delivered before the drug is washed in and the critical pathway is stimulated (i.e. strong-before-strong-with-drug). This is possible due to the fact that the heterosynaptic plasticity behind the STC hypothesis is independent of the order of stimulation (Frey and Morris, 1998b; O'Carroll and Morris, 2004). The conclusions based on the fate of the synapses stimulated under the influence of the drug are the same as those described above.

Figure 1.2 Three possible causes in the failure of maintaining LTP.

The initial ingredient in the set of experiments described in this chapter and used in this thesis is the use of a compound capable of blocking the maintenance of LTP. For all the available drugs capable of achieving this block, we can ask the question of what is their mode of action within the STC hypothesis.

This figure makes use of the inverted pyramid used in figure 1.1 to depict the pathways necessary for the maintenance of LTP. Pathways only necessary for tag-setting (red tag, pathway) or PRP availability (blue PRPs, pathway) or pathways involved in both (purple base).

There are three options depicted in the cartoons:

1) **The drug does not allow for the maintenance of LTP because it interferes with the pathways leading to the synthesis of the PRPs (blue pathways 'crumble').**

2) **The drug blocks the setting of the tag or its ability to capture the required PRPs, which are synthesized nonetheless (red pathways blocked and tag is not set or maintained).**

3) **The drug is capable of preventing both the setting of the tag and the synthesis of PRPs (purple basis pathways fail to sustain the requirements for the maintenance of synaptic plasticity).**

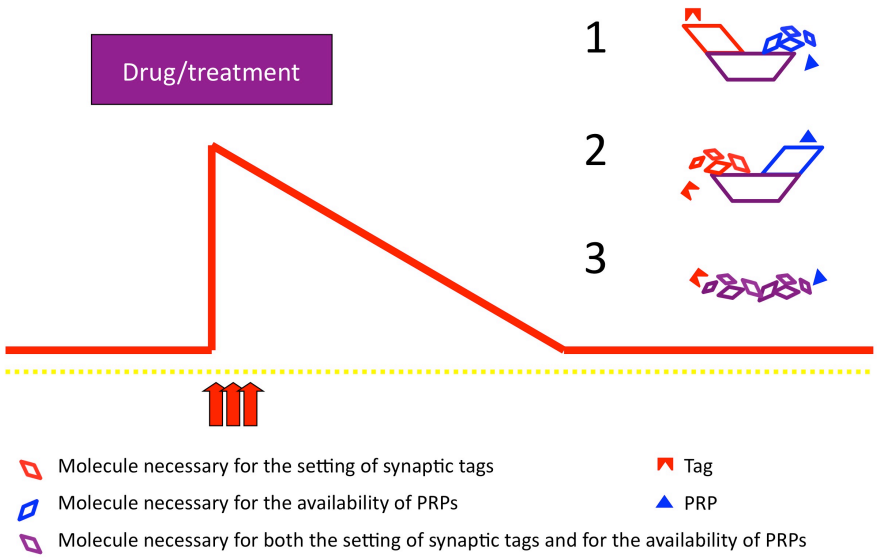


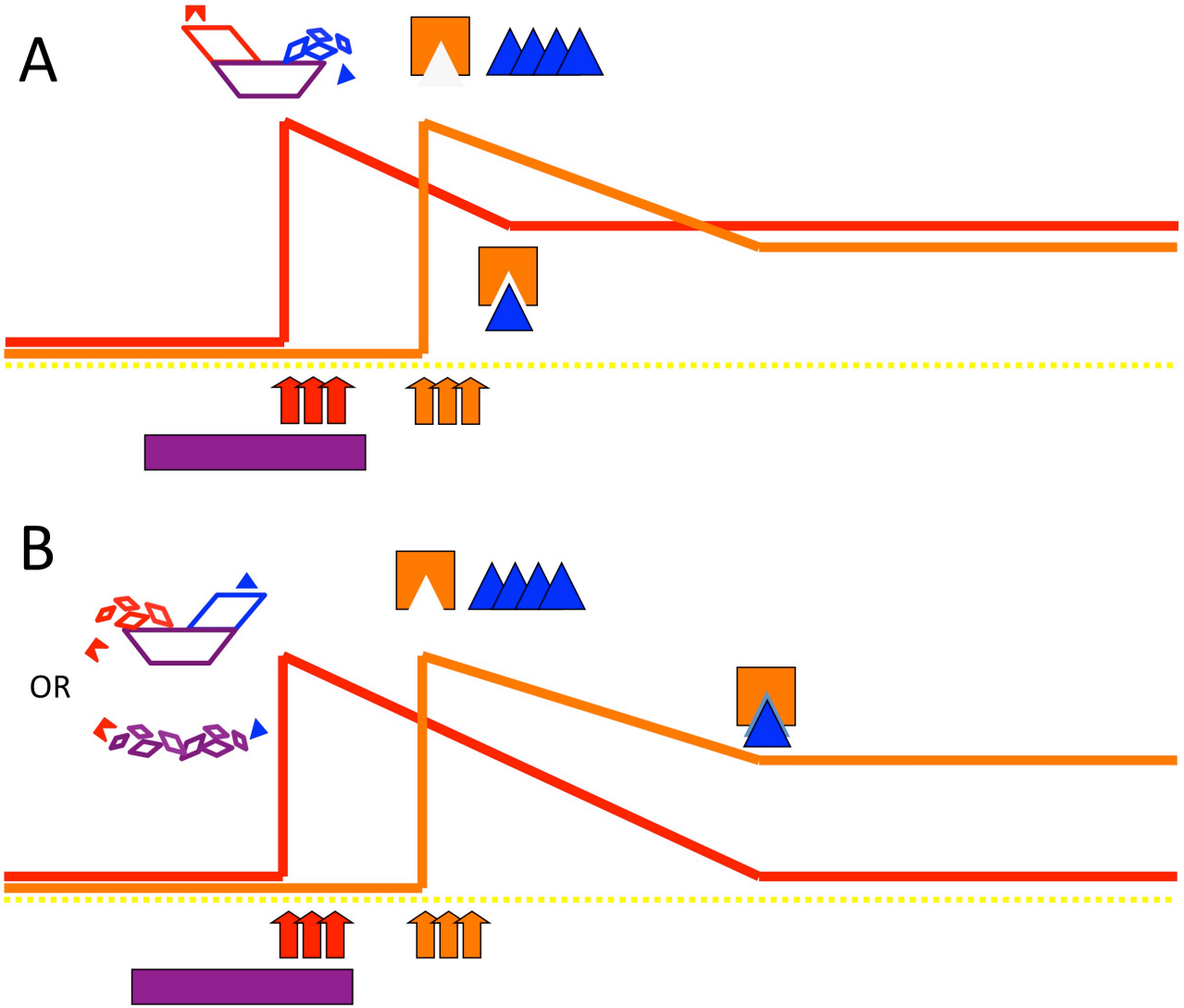
Figure 1.3 Two possible outcomes for a test for 'PRP-block' experiment

This experiment involves the delivery of strong stimulation to one set of synapses (see methods for details) while in the presence of the compound of interest, and after washout or removal of the drug, another strong stimulation this time onto a separate set of axons convergent onto the same neuronal population as the first stimulation. There are two possible outcomes of this experiment.

A) The potentiation elicited under the presence of the drug is maintained (red). Since this hypothetical drug is successful in blocking LTP when elicited on its own (Figure 1.2) we can conclude that the effects of the drug can be rescued by the consequences of the second tetanization (orange). Whatever the drug was interfering with can be provided by the stimulation of independent synapses and we can also conclude that local, synapse-specific mechanisms (i.e. tags) were left intact.

B) The synapses stimulated under the effects of the drug fail to maintain their potentiation. This happens even while the other stimulated pathway has no trouble sustaining L-LTP. All the ingredients for the successful maintenance of LTP are synthesized and utilized by the second pathway stimulated outside the influence of the drug (orange). In this case, we can conclude that the drug has inhibited the local requirements for the maintenance of LTP (tag). With this result, however, it is impossible to conclude whether the drug did also block the signaling mechanisms leading to the synthesis of PRPs.

The inverted pyramid drawings depict the possible reasons for both outcomes (A or B). Only outcome A is conclusive. Symbols as figure 1.2 with colours matching pathway of origin.


A
B
OR

Test for 'tag block' (Strong before weak, Figure 1.4)

As with the strong-before-strong experiments, this type of experiment uses an inhibitor of a candidate molecule that has been shown to be capable of blocking L-LTP if present at the time of induction. If the inhibitor can be washed-out out of the preparation, the strong tetanus can be delivered to one pathway under its influence, then washed out, and later a weak tetanus can be applied to the second pathway. This weak tetanus, delivered on its own, produces E-LTP due to the lack of synthesis of PRPs.

If in this experiment, the weakly tetanized synapses succeed in showing L-LTP while the strongly tetanized decay to baseline levels of synaptic efficacy, the drug must be interfering with the tag but not with availability of the products of the *de novo* protein synthesis. In other words, the fact that the weakly stimulated synapses maintain L-LTP requires their successful use of PRPs. These PRPs could only have been produced by the strong tetanus even though it was delivered to the first pathway under the influence of our theoretical inhibitor. Since the strongly tetanized synapses still fail to show L-LTP, even with PRPs available to the other pathway, their synapses must have dysfunctional tags. This result is definitive in pinpointing a tag-specific role to whatever molecule or process the theoretical inhibitor was interfering with (Figure 1.4).

This conclusion is complicated by the possibility that failure to show L-LTP in the strong pathway to be a result of a disruption in the expression of LTP. If the expression of synaptic plasticity were independent of the tag, blocking the expression of LTP would have the same effect in the strongly tetanized pathway as the block of the tag. The possible distinction between the expression of synaptic plasticity and the setting of the tag will be discussed in chapter 11.

The alternative result of the strong-with-drug-before-weak experiment involves both the strong and the weakly stimulated pathways failing to show L-LTP. In this case, we can claim with certainty that the inhibitor prevents the synthesis and availability of PRPs; otherwise the weakly stimulated synapses would have made use of them. However, in a reversed scenario to that described in the strong-before-strong experiments, this result is not conclusive as to whether the inhibitor is

specifically acting on the molecular pathways leading to the synthesis of PRPs or whether it is also blocking the setting of the tag (Frey and Morris, 1997; O'Carroll and Morris, 2004).

If the drug cannot be washed out of the preparation, weak before strong protocol with a drug infused right before the strong tetanus can still inform us about the mechanisms that set the tag, although in this experiment the drug could interfere with the already set tags in the weakly tetanized synapses.

The combination of strong-before-strong and strong-before-weak experiments should be definitive in determining necessary roles in the sequence of events leading to STC to candidate molecules. The experimental application of these theoretical experiments can be seen in chapters 6 and 7 of this thesis. First, however, one needs to identify these candidate molecules and the drugs that can allow the experiments described in this section. Fortunately, the literature offers us a window into what may be necessary for the setting of the tags, what may be necessary for the availability of PRPs and what molecular actors may be necessary for these two requirements for the maintenance of synaptic plasticity.

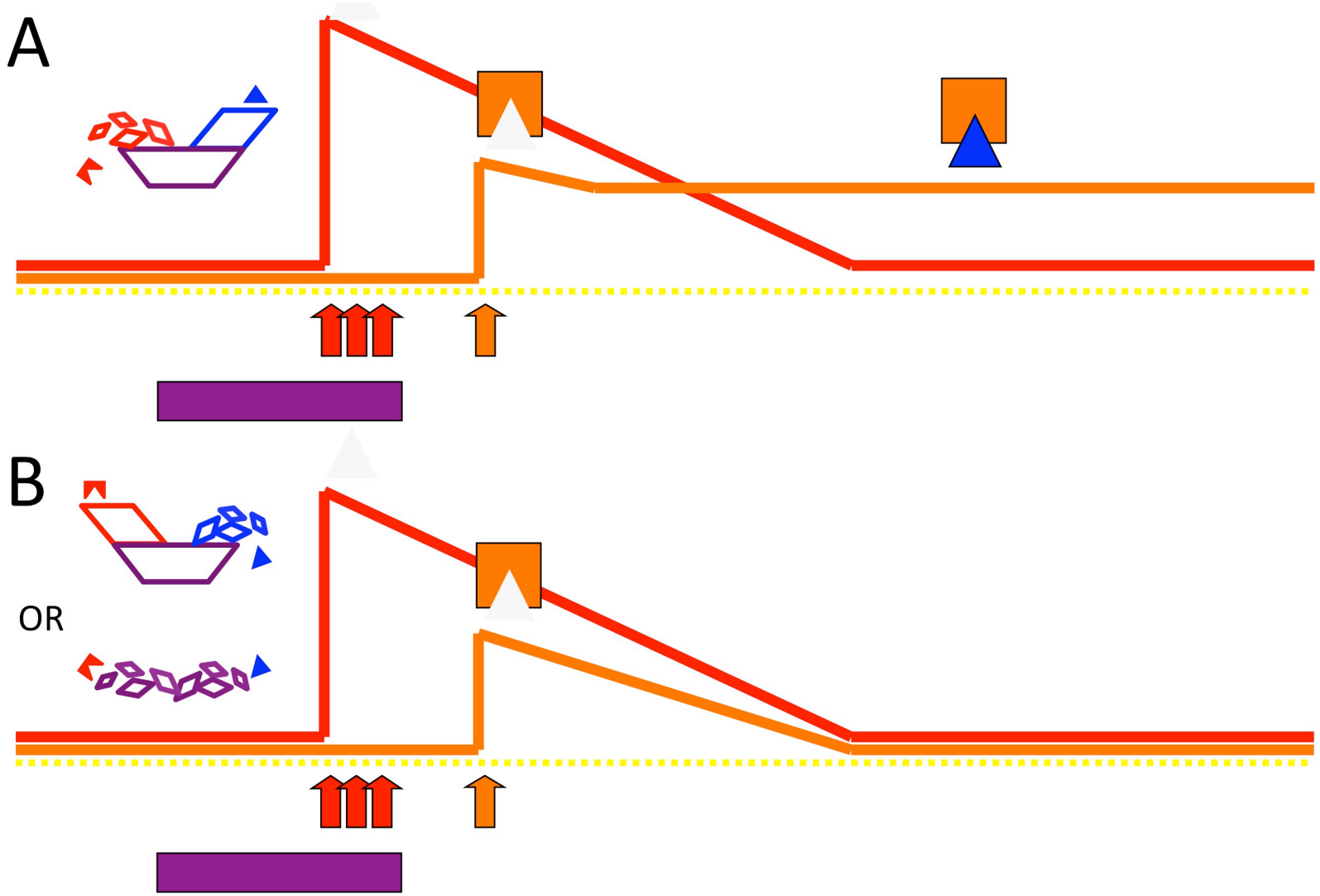
Figure 1.4 Two possible outcomes for a test for tag block experiment

This experiment involves the delivery of strong stimulation to one set of synapses (see methods for details) while in the presence of the compound of interest, and after washout or removal of the drug, the delivery of weak stimulation onto a separate set of axons convergent onto the same neuronal population as the first stimulation. There are two possible outcomes of this experiment. In every case, the strongly tetanized synapses are expected to fail to maintain their potentiation. The informative results are given by the outcome of the stimulation of the weak pathway (orange).

A) The weakly stimulated pathway is capable of maintaining its potentiated state. The stimulation used to trigger this weak potentiation has to be known beforehand to be capable only of eliciting E-LTP. If however, under the conditions of this strong-before-weak experiment, the weakly stimulated synapses are capable of showing L-LTP, it means that somehow they are finding and making use of PRPs. The only event capable of having made the PRPs available has to be the strong stimulation delivered under the presence of the drug of interest. Therefore, even though the drug is capable of blocking L-LTP, it does allow for the synthesis of PRPs. One can conclude then, that the drug is acting by preventing the setting or the action of the tag.

B) The weakly stimulated pathway only shows E-LTP, as if given on its own. These results are less informative since they do not allow us to conclude whether the drug blocked the L-LTP in the strongly tetanized pathway by interfering with the pathways leading to the synthesis of PRPs or whether the tags were also missing.

The inverted pyramid drawings depict the possible reasons for both outcomes (A or B). Only outcome A is conclusive. Symbols as figure 1.2 with colours matching pathway of origin.


A
B
OR

1.5 The setting of the synaptic tag (a sequence of necessary events)

The 'Tag' can be defined as the process (and the molecules that enable it) that allows for the targeting of the products of protein synthesis to be captured and used by stimulated synapses.

The tag has to satisfy separate criteria:

- Induced in a protein synthesis-independent manner (Frey and Morris, 1997),
- Lifetime of 1-2 hours (32 degrees) (Frey and Morris, 1998b),
- Inducible by stimulation parameters that trigger both E-LTP/E-LTD and L-LTP/L-LTD (Sajikumar and Frey, 2004a),
- Input-specific and physically immobile (Frey and Morris, 1997),
- Interacts with the proteins required for L-LTP/L-LTD to facilitate capture (Kelleher et al., 2004b),
- Is capable of being reset by specific patterns of neuronal activity (Bortolotto et al., 1994; Sajikumar and Frey, 2004a; Young and Nguyen, 2005).

Molecules necessary for the setting of the tag should be susceptible to detection through a test for tag block as described above. However, if the tag is a 'state of receptability' of the synapse to the PRPs, there may be different types of processes in which it depends.

What follows is a review of possible types of molecules involved in either the weakening or the stabilization of the synapse, possibly in a way independent of those pathways that lead to cellular synthesis of proteins. These molecules, due to their local role in synaptic plasticity, can be predicted to be necessary for the setting of synaptic tags. Theoretically, every one of the molecules reviewed could be tested with the tag-blocking experiments.

Although these molecules will be explained one by one in this chapter of the thesis, Chapter 11 will combine the information gathered below into a step by step sequence of events in an attempt to providing a clearer view of the processes involved in synaptic tagging and capture.

1.5.1 NMDAR activation is necessary for both tag setting and PRP availability.

Formation of the synaptic tag requires activation of NMDARs (Barco et al., 2002). The blocking of NMDAR with APV prevented LTP and the tagging of synapses in the hippocampus (Barco et al., 2002) and the targeting of PRPs to activated dendrites in the dentate gyrus (Steward and Worley, 2001). However, these experiments were insufficient to assess whether NMDAR are necessary to synthesize the PRPs. This is because the experiments conducted by Barco et al. in 2002 tell us about the necessity of certain molecules for the setting of the tag, without elucidating whether these molecules are also necessary to send the signal that engages protein synthesis. Experiments with the drug given during the weak tetanus do not discern between a tag-specific molecule and a molecule also necessary for the synthesis of PRPs. The more informative experiment involves the presence of APV during the strong tetanus to one pathway followed by drug washout before weak tetanisation of the second pathway (O'Carroll and Morris, 2004). In this case, the weakly tetanised pathway, with its tagged synapses, fails to find and capture PRPs, which leads to the conclusion that the presence of APV during the strong tetanus to the first pathway blocked whatever signalling cascade was required to engage the synthesis of PRPs. This is one of the possible outcomes of a 'tag-blocking' experiment and shows that NMDARs are involved and necessary in both the setting of the tag and the synthesis of PRPs. This result is not too surprising since a blockage of NMDAR function would limit the entry of Ca^{2+} ions into the postsynaptic terminal and we shall see in chapters 6 and 7 how calcium signalling is required for both the setting of tags and the availability of PRPs. There is, however, some new information that can be learned from these experiments since they imply that no matter what the role of other sources of calcium influx are (VGCC, Internal stores, …) they are not sufficient, without NMDAR activation to set tags or to make PRPs available.

1.5.2 Protein kinases

Calmodulin (CaM) may read out the different temporal profiles in calcium influx that determine the direction of synaptic change (DeMaria et al., 2001). Key targets of

CaM are the CaM-dependent kinases, especially CaM-dependent protein kinase II (CaMKII) and the CaM-dependent phosphatase PP2B (calcineurin). CaMKII is a broad range kinase that regulates many neuronal functions (Erondu and Kennedy, 1985; Braun and Schulman, 1995; Yamauchi, 2005). In the CA3 to CA1 pathway of the hippocampus, CaMKII activation by Ca^{2+}/CaM is necessary at the time of LTP induction (Malenka et al., 1989), but not during LTP maintenance (Malinow et al., 1989; Otmakhov et al., 1997; Bortolotto and Collingridge, 1998; Chen et al., 2001). This is possible due to the fact that the 12 subunits that form this holoenzyme have the ability to autophosphorylate each other in the presence of calcium-calmodulin, and thus remain active (Kuret and Schulman, 1984; Yamauchi and Fujisawa, 1985). The CaM kinases consist of an N-terminal catalytic domain, a regulatory domain, and an association domain. In the absence of Ca^{2+}/calmodulin, the catalytic domain is autoinhibited by the regulatory domain, which contains a pseudosubstrate sequence. Firstly, the required binding of calmodulin in the presence of Ca^{2+} disrupts the autoinhibitory domain of CaMKII. Then, the phosphorylation of threonine 286 (Thr^{286}) increases the affinity for calmodulin creating a state where CaMKII traps calmodulin even after the end of a calcium transient (Meyer et al., 1992). This has two effects: An increase in affinity for the calmodulin complex, prolonging the time the kinase is active and continued activation of the phosphorylated kinase complex even after the calmodulin complex has dissociated from the kinase complex, which prolongs the active state even more. It has been proposed that this property may enable CaMKII to act as a switch capable of maintaining changes in synapse efficacy (Lisman and Goldring, 1988; Miller et al., 2005). In this state, CaMKII binding to the NR2B subunit of the NMDAR becomes persistent (Bayer et al., 2006) keeping it in the postsynaptic density and decreasing the inhibitory effect of some phosphatases on CaMKII (Strack et al., 1997a; Fox et al., 2006). This switch quality could account for tag setting or for the capture of newly synthesized PRPs.

Once in the PSD, CaMKII phosphorylates more than 28 substrates (PSD-95, tubulin, neurofilaments, glutamate receptor subunits, among others) (Yoshimura et al., 2000). CaMKII enhances Ca^{2+} influx through the NMDAR (Kitamura et al., 1993). CaMKII also increases the AMPAR GluR1 subunit's conductance (Derkach et al., 1999). AMPAR trafficking is also affected through CaMKII phosphorylation

of the AMPAR associated protein stargazing, which promotes the incorporation of AMPAR into the PSD (Tomita et al., 2005). CaMKII will also regulate the Ras-GTPase SynGAP and in this way affect cofilin and actin binding (Carlisle et al., 2008b), AMPAR insertion (Krapivinsky et al., 2004), and also the MAPK pathway (Cobb and Goldsmith, 1995; Rumbaugh et al., 2006). Interestingly, however, there is a great deal of confusion concerning the consequences of CaMKII phosphorylation of SynGAP (Chen et al., 1998; Kim et al., 1998; Song et al., 2003; Oh et al., 2004).

CaMKII also has important presynaptic roles. One type of presynaptic change is expressed as changes in the properties of VGCC and this requires their modulation by CaMKII. Vesicle release relies on the interaction between Syntaxin and SNAP-25 with the VGCC. The synprint peptide binds to Syntaxin preventing this interaction unless active CaMKII phosphorylates synprint thereby blocking its inhibition of the release machinery (Yokoyama et al., 2005). In this way, presynaptic CaMKII activation may contribute to synaptic plasticity.

With all these possibilities in mind, this thesis assesses the role of CaMKII as a candidate for a tag-specific molecule (see chapter 6). My results support a specific role of CaMKII in the setting of tags and will be discussed in chapter 11.

1.5.3 Extracellular Matrix role in tag setting

Besides the persistent activity of certain kinases being a requirement for the setting of local tags, structural changes in the dendritic spine could also be necessary and specific for a functional tag. A series of molecules have been shown to play important roles in the maintenance of LTP and below are some good candidates for tag-blocking experiments.

Arachidonic acid (AA)

AA is an unsaturated fatty acid that satisfies several requirements for a retrograde messenger (Bliss and Collingridge, 1993). Glutamate activation of mGluR G protein activity engages Phospolipase A2 (PLPA2) that releases AA from membrane phospholipids. As a retrograde messenger, AA can increase the release of glutamate in the presynaptic terminal (Williams et al., 1989) and inhibit the re-uptake by glial cells (Arai and Lynch, 1992). At the same time, AA can potentiate NMDA currents

(Miller et al., 1992) Inhibition of PLPA2, an enzyme that liberates arachidonic acid from phospholipids (Clements et al., 1991), blocks the induction of LTP in the CA1 region of the hippocampus (Okada et al., 1989). AA and PLPA2 are candidate molecules for a 'tag block' experiment. If one can reversibly block the release of AA (by inhibiting the secretion of PLPA2 (Farooqui et al., 2006)), one could check that AA is necessary for the setting of the tag but not the pathways leading to PRP synthesis through a 'tag-block' experiment.

Laminin, plasmin and tissue-type plasminogen activator

Laminin-mediated cell-ECM interaction may be necessary for the maintenance of LTP (Nakagami et al., 2000). Laminin is a substrate of the protease plasmin. In acute slices, application of plasmin during tetanic stimulation facilitates the induction of LTP (Mizutani et al., 1996). Specifically, 100 nM Plasmin enhanced short-term potentiation (STP) by a weak tetanus (10p 100Hz) and that 100 nM Leupeptin (protease inhibitor) blocked LTP by a strong tetanus (100p 100Hz). The effect of plasmin is only seen when infused during induction, not during maintenance. Endogenous plasmin may be released in response to a strong tetanus but not to an STP-inducing tetanus. Plasmin and its actions could be involved in the tag processes. Plasmin degrades laminin but not fibronectin and type IV collagen.

Tissue plasminogen activator (tPA) is a secreted serine protease that converts the proenzyme plasminogen to plasmin, a fibrinolytic enzyme. Since tPA regulates plasmin, transgenic mice over expressing tPA have increased and prolonged hippocampal LTP and improved performance in spatial orientation learning tasks (Madani et al., 1999). tPA is induced as an immediate early gene during LTP (Qian et al., 1993). On the other hand, mice lacking the tPA gene show a selective defect in L-LTP (Frey et al., 1996; Huang et al., 1996)

tPA activates plasmin that then converts precursor proBDNF into mature BDNF (mBDNF). And mBDNF application rescues the L-LTP in slices treated with anisomycin (40microM) (Pang and Lu, 2004). Furthermore, application of mBDNF converts E-LTP into L-LTP and tPA application during tetanic stimulation enhances the late phase of LTP in rat HPC slices (Baranes et al., 1998). Some argue that the effect of plasmin is not likely to be mediated by proteolytic activity of plasmin since

it would cause an irreversible change in the target protein (Mizutani et al., 1997). They also show that the enhancement of STP is probably due to a decrease in inhibition by GABA-induced chloride currents, maybe by increasing the concentration of intracellular Ca^{2+}. If the effects of plasmin are so general, then its inhibition by leupeptin should block both the tag and the PS pathway. Still, the local actions of plasmin on BDNF make it a possible tag molecule and a worthy candidate of a 'tag-block' experiment.

Somewhere else in the ECM, Heparin-binding growth-associated molecule (HB-GAM) is an ECM associated protein involved in synaptogenesis whose over expression allows mice to learn the water maze faster (Pavlov et al., 2002). Synd3 acts as a receptor for HB-GAM and interacts with intracellular cytoskeleton-regulating molecules such as cortactin. Cortactin is a Shank binding protein, providing a link between the postsynaptic density and the actin cytoskeleton. This can be another molecular cascade necessary for the conformational changes required to set the tag.

Other ECM molecules are reviewed in Dityatev 2003 (Dityatev and Schachner, 2003). Neuropsin, for instance, is an ECM protease and its inhibition disrupts E-LTP, but not L-LTP, in a NMDAR and calcium influx independent manner (Komai et al., 2000).

As long as their roles are local, it can be predicted that they will not be necessary for the synthesis of diffusible PRPs and therefore they are candidates for a specific role in the setting of the tag.

1.5.4 Cell adhesion molecules

Cell Adhesion Molecules (CAMs) are proteins located on the cell surface involved with the binding with other cells or with the extracellular matrix (ECM) in the process called cell adhesion.

There is the suggestion that LTP induction involves a perforation of the postsynaptic densities and a redistribution of the postsynaptic receptors and presynaptic active zones (Geinisman, 1993; Buchs and Muller, 1996). This may open the possibility of interfering with the expression of LTP by blocking these synaptic

modifications in a way that does not interfere with the synthesis of plasticity proteins.

CAMs can stabilize the changes that occur during synaptic plasticity by regulating spine head size. Also, stimulation leading to LTP increases the synaptically localized NCAM112 and cadherin126. The expression of LTP may require the destabilization of the ECM to allow structural changes. The CAMs described below would have a major role in this process (Dalva et al., 2007). There is the immunoglobulin superfamily, integrins and cadherins (Benson et al., 2000; Balschun et al., 2003).

Immunoglobulin superfamily of CAMs

Neural Cell Adhesion Molecule (NCAM) and polisialic acid-NCAM (PSA-NCAM) are candidates to a have a role in tag-setting. Endoneuraminidase (Endo-N) cleaves the a-2-8-linked polisialic acid residues that associate with NCAM and when this happens, LTP and LTD is inhibited despite NMDAR responses being unaltered (Muller et al., 1996). NCAM-knockout mice show impaired spatial learning when tested in the Morris water maze while similar deficits in spatial learning are evident in rats following enzymatic removal of sialic acid (with endo-neuraminidase N (endo-N)) from PSA-NCAM (Hallenbeck et al., 1987; Becker et al., 1996; Shen et al., 1997).

Cell adhesion can be weakened by internalization or glycosylation of NCAM as seen after the serotonergic induction of facilitation in Aplysia (Bailey et al., 1992; Mayford et al., 1992). In chicks, antibodies against L1 and NCAM prevent memory consolidation when added just after encoding and 4-6 hours later only (Rose, 1995). In rats, the intraventricular injection of anti-NCAM (Doyle et al., 1992b) or a synthetic peptide that prevents NCAM internalization during or 6–8 h after training inhibited memory consolidation in a passive avoidance task (Foley et al., 2000). Consolidation was also distinctly attenuated when anti-L1 or anti-NCAM was chronically infused into the ventricle during spatial learning in a Morris water maze (Arami et al., 1996). Interfering with L1 or NCAM function in brain slices through bath application of antibodies against these cell adhesion molecules did indeed prevent the development of hippocampal long-term, but not short-term, potentiation (Luthl et al., 1994). In theory, CAMs would first need to destabilize the synapse to

allow for LTP expression while later, other CAMs would stabilize and lock the new changes brought by synaptic plasticity. Fitting with this theory, there is an increase in the synthesis of these glycoproteins and an increase in their glycosylation, immediately and 4-6 hours after training. NCAM glycosylation and/or internalization are necessary for memory consolidation. Adhesion properties of L1 and NCAM can be efficiently regulated through glycosylation. In the brain, PSA seems to be selectively associated with NCAM. It is thought that the negatively charged clouds of PSA sugar groups at pre- and postsynaptic sites reject each other and thus lead to a measurable increase in size of the synaptic cleft. The resulting decrease in adhesive strength appears to be necessary for the remodelling of synapses so that a long-lasting memory trace in the form of a facilitated transmission ensues. Increased polysialation of NCAM has been observed 12 and 24 h after one-trial passive avoidance learning (Doyle et al., 1992a). When PSA was continually clipped off NCAM with intracranially injected endoneuraminidase spatial learning in rats was attenuated. The same enzyme when added to brain slices *in vitro* also blocked hippocampal long-term potentiation (Becker et al., 1996). All this knowledge suggests that PSA-NCAM interactions could have a necessary local role for the maintenance of synaptic plasticity and that these actions would be independent of those required for the synthesis of PRPs. Disruption of PSA-NCAM during a 'tag-block' experiment could reveal a necessary role of this cell-adhesion molecule in the setting of the tag.

Recently, however, additional functions have been attributed to NCAM (reviewed by Hinsby et al 2006). NCAM is both a cell-cell adhesion molecule and a signalling receptor. Because of its signalling properties, interfering with NCAM may also affect the synthesis of PRPs. If so, a 'tag-block' experiment should not show a rescue of the weak tetanus. Any of the outcomes of the hypothetical experiment would nonetheless be highly informative.

Another CAM, L1, is a CAM that localizes in synapses of CA1 and is cleaved by the serine protease, neuropsin (Matsumoto-Miyai et al., 2003). This cleavage is neural activity dependent and it is required in order to see E-LTP (Tamura et al., 2006b). The question is very similar to that proposed for PSA-NCAM: is L1 cleavage required for just the setting of the tag or does the consequent disruption in signalling block the pathways leading to the synthesis of PRPs?

Integrins

Integrins are internal membrane proteins in the plasma membrane of cells. Integrins are heterodimeric glycoproteins that interact cytoplasmically with actin via talin, alpha-actinin or vinculin. Extracellularly, they recognize many matrix proteins. Alpha 8 and Beta8 are concentrated at some postsynaptic densities (Benson et al., 2000) They play a role in the attachment of a cell to the extracellular matrix (ECM) and in signal transduction from the ECM to the cell. The integrin antagonist GRGDSP has been shown to reverse LTP in slices when applied during but not after LTP induction (Staubli et al., 1998). This suggests a necessary role for integrin signalling at the time of induction. Is the role of integrins in synaptic plasticity restricted to the setting of the tag?

Mice with genetically reduced expression of alpha3 integrin fail to maintain long-term potentiation (LTP) generated in hippocampal CA1 neurons. Mice with reduced expression of the alpha3 and alpha5 integrins are defective in paired-pulse facilitation and in hippocampal LTP and spatial memory in the water maze but have normal fear conditioning (Chan et al., 2003). Are these integrins necessary for the setting of local tags or do their roles in signalling engage the synthesis of PRPs?

Cadherin

Cadherins are a class of transmembrane proteins that play important roles in cell adhesion whereby they ensure cells within tissues are bound together. They are dependent on calcium (Ca^{2+}) ions to function, hence their name. Cadherin is required for activity induced spine remodelling (Okamura et al., 2004) and its blockage with antibodies impairs L-LTP but not E-LTP (Bozdagi et al., 2000). Activation of NMDAR reduces the rate of endocytosis of N-cadherin, resulting in its accumulation in the plasma membrane. The stabilization of surface N-cadherin molecules blocks NMDAR-dependent LTD (Tai et al., 2007). Is the impairment in L-LTP after cadherin block due to the lack of functional tags, PRPs, or both?

1.5.5 Tag setting changes the postsynaptic density (PSD)

The PSD is a specialization of the cytoskeleton at neuronal synapses that was originally identified as an electron-dense region at the membrane of a postsynaptic neuron, as viewed by electron microscopy. PSDs are usually comprised of L-glutamate neurotransmitter receptors, molecular scaffolding molecules, cell adhesion molecules and a diverse set of other signalling proteins. PSDs vary in size and composition among brain regions. Many of the PSD proteins contain PDZ domains. Interestingly, PSD95 -/- mice show enhanced LTP (Migaud et al., 1998; Carlisle et al., 2008a) The induction of synaptic plasticity, expressed as the modification in the number of glutamatergic receptors, or as their turnover at the plasma membrane, will require modifications of the PSD and the molecules found there have become candidates for necessary roles in synaptic tagging and capture. Could processes leading to the tag have an effect on the postsynaptic density (PSD)?

Shank/Homer/SPAR

These are three proteins of the PSD capable of interacting with many other proteins, and connecting the NMDAR and mGLUR with the actin cytoskeleton (reviewed by Michael Ehlers in 2002 (Ehlers, 2002)). They have a role in spine morphogenesis that may be very important for learning and memory. NMDA receptors are linked to intracellular cytoskeletal and signalling molecules via the PSD-95 protein complex. A family of postsynaptic density (PSD) proteins, termed Shank, may function as a scaffold protein in the PSD, potentially cross-linking NMDA receptor/PSD-95 complexes and coupling them to regulators of the actin cytoskeleton like cortactin (Naisbitt et al., 1999). Following glutamate stimulation, cortactin but not Shank is moved out of the spine into the dendritic shaft, allowing for actin reconfiguration (Hering and Sheng, 2003).

PSD-95 couples NMDARs to the Ras GTPase-activating protein SynGAP and SynGAP seems to be necessary for activation of multiple downstream signalling pathways since mutant mice without it showed reduced LTP (Komiyama et al., 2002). What would be the outcome of a ‘tag-block’ experiment where the action of SynGAP has been compromised?

SPAR (Spine-associated Rap guanosine triphosphatase activating protein), a postsynaptic actin regulatory protein, is phosphorylated and eliminated by serum-inducible kinase (SNK). This elimination causes the loss of mature dendritic spines through depletion of PSD-95 and Basoon clusters. SNK is inducible by synaptic activity at the mRNA level. So, SNK with SPAR are activity-driven changers of the molecular composition and morphology of spines (Pak and Sheng, 2003). Maybe SNK is translated after synaptic activity to inactivate SPAR, therefore allowing for PSD restructuring. The SPAR-SNK interaction could also be the target of a test for tag block.

1.5.6 Changes in actin cytoskeleton are required for a functional tag

The shape of a dendritic spine and the conformation of its PSD are dependent on the actin cytoskeleton. Actin is a globular structural protein that polymerizes in a helical fashion to form an actin filament. These microfilaments form the cytoskeleton - a three-dimensional network inside a eukaryotic cell. Actin filaments provide mechanical support for the cell, determine the cell shape, enable cell movements (through lamellipodia, filopodia, or pseudopodia); and participate in certain cell junctions, in cytoplasmic streaming and in contraction of the cell during cytokinesis. The actin cytoskeleton interacts with scaffolding proteins (Carlisle and Kennedy, 2005). Current evidence favours a proposed model in which two pools of actin filaments, one stable and the other dynamic, support both persistent spine structure and rapid spine motility. Potential functions of spine motility and dynamic actin include regulated protein scaffolding, retrograde signalling and synapse stabilization (Halpain, 2000). Spines can undergo rapid changes after different types of stimulus (electrical, behavioural and hormonal). Although Sorra et al. report no changes in synapse number and size 2 hours after LTP in CA1 (Sorra and Harris, 1998), other older literature describe alteration in spine shape after intense synaptic activity (Fifkova and Van Harreveld, 1977; Lee et al., 1980; Desmond and Levy, 1983; Chang and Greenough, 1984). Recently it has been reported that theta stimulation polymerizes actin in dendritic spines (Lin et al., 2005). The inhibition of actin function blocks synapse formation and interferes with long-term synaptic plasticity

(Krucker et al., 2000; Zhang and Benson, 2001). The use of drugs that prevent actin reconfiguration (cytochalasin D, latrunculin A, or cytochalasin B) blocks L-LTP. Structural remodelling of synapses in response to physiological activity requires reorganization of the actin network (Huntley et al., 2002) and the inhibition of actin function blocks synapse formation and interferes with long-term synaptic plasticity (Krucker et al., 2000; Zhang and Benson, 2001). Not surprisingly, NMDAR opening is required for actin reconfiguration at the synapse while VGCC activation is necessary for actin-cytoskeleton remodelling in the soma (Furuyashiki et al., 2002).

The clearest evidence for a role of F-actin in LTP comes from a study in the dentate gyrus *in vivo* where quite strong stimulation (up to 2400 HFS pulses) elicited LTP lasting for 5 weeks and immunohistochemisty performed on those brains revealed long-lasting actin polymerisation restricted to the dendritic regions that had received the potentiating input (Fukazawa et al., 2003). This polymerisation was in part due to the phosphorylation and consequent inactivation of ADF/cofilin, an actin-binding depolymerising protein (Pantaloni et al., 2001).

Interestingly, microtubules do not seem to be necessary for LTP (Vickers and Wyllie, 2007). A valid prediction from the literature is that molecules involved in actin polymerisation may be necessary to allow local changes in the spine (i.e. setting of tags) while biochemical cascades leading to the synthesis of PRPs may remain unaltered. Adducin, for instance, interacts with the actin cytoskeleton in a calcium- and cAMP-dependent manner. High levels of adducin mRNA can be found in the hippocampus and adducin-KO mice show impaired LTP and performance in fear conditioning and water maze tasks. (Rabenstein et al., 2005). The role of adducing in STC could also be assessed with the type of experiments described above (tag-block and PRP-block experiments).

1.5.7 Local translation of mRNAs

There is a transcription-independent, translation-dependent phase of LTP during the first 60-90 min following tetanisation (Kelleher et al., 2004b).. This translation could happen locally in the dendrites since polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP (Ostroff et al., 2002) and there is a rich repertoire of mRNAs that can be identified as translated in dendrites (Poon

et al., 2006). The relevance of local translation derives from the types of molecules that have been found to be locally synthesized: AMPAR subunits GluR1 and GluR2 can be synthesized in dendrites after stimulation (Ju et al., 2004).

Bailey, Kandel and Si (2004), propose a model to explain how a cell-wide process can be used in a synapse-specific way through the control of mRNA synthesis (Bailey et al., 2004). In Aplysia cultured cells, repeated application of 5-HT to one synapse produces a CREB-mediated, synapse-specific long-term facilitation that is accompanied by the growth of new synaptic connections and persists for at least 72 h (Casadio et al., 1999). But, in addition to CREB-mediated transcription, one needs a 'marking' signal produced by a single pulse of 5-HT applied to the synapse. Cytoplasmic polyadenylation element binding protein (CPEB) is capable of activating dormant mRNAs through the elongation of their polyA tails. The induction of CPEB coincides with the polyadenylation of neuronal actin, and blocking CPEB locally at the activated synapse blocks the long-term maintenance of synaptic facilitation but not its early expression at 24 h (Si et al., 2003a). The mouse isoform is CPEB-3 and it is induced by dopamine (Theis et al., 2003) which suggests that PKA activation might be necessary for tag setting. Among other mRNAs, N-actin and alpha tubulin are present in the peripheral population of mRNAs (Moccia et al., 2003). Thus, CPEB might contribute to the stabilization of learning-related synaptic growth by controlling the synthesis of both the structural molecules such as tubulin and N-actin and the regulatory molecules such as CaMKII. One possible answer to how a population of unstable molecules can produce a stable change in synaptic form and function comes from the finding by Si et al. (2003b) that the neuronal isoform of CPEB shares properties with prion-like proteins (Si et al., 2003b). CPEB is a prion and unlike the known prion proteins where the dominant state is the inactive form of the protein, surprisingly, in the case of *Aplysia* CPEB, the dominant form is the active form of the protein capable of activating translationally dormant mRNAs (Si et al., 2003a). CPEB could then show the switch like properties that have been proposed for CaMKII and PKMζ.

EF-1A

Eukaryotic elongation factor 1A (eEF1A), which binds and transports aminoacyl-tRNA to the A site of the ribosome, is another molecule with an enabling role for the

synthesis of PRPs. Dendritic elongation factor 1A mRNA is dendritically translated after induction of L-LTP, through rapamycin (mTOR) pathway and the protein is present for about 3h (Tsokas et al., 2005). EF-1A is also translated in response to treatments that elicit LTD (Huang et al., 2005). There are lots more of these translation factors (Klann et al., 2004) and although they are necessary for protein synthesis, the products of this dendritic synthesis could be involved in the stabilization of a synaptic change (Govindarajan et al., 2006; Richter and Klann, 2009).

1.5.8 Protein degradation

The ubiquitin-proteasome system interacts with many of the proteins mentioned above as candidates for a role in tag setting. Its activation or inhibition could also be part of the tagging system itself (Hegde, 2004). Right after LTP induction, there is an increase in both protein synthesis and protein degradation (Karpova et al., 2006). The proteasome inhibitor MG132 significantly reduces the field EPSP slope potentiation and LTP maintenance without acutely affecting basal synaptic transmission (Karpova et al., 2006). In another study, blocking the proteasome disrupts the maintenance of L-LTP. However, simultaneous inhibition of the proteasome and the synthesis of proteins elicits an LTP indistinguishable from controls (Fonseca et al., 2006b). These findings support the view that there is a constant cycle of protein translation and degradation. Does a functional tag, capable of capturing newly synthesized PRPs, require the degradation of certain proteins at or around the synapse? And is the role of the proteasome restricted to these local changes? If so, a tag-block experiment should show a failure to show LTP in the pathway tetanized under the influence of a proteasome inhibitor while the weak tetanization delivered to an independent set of synapses after the drug washes out should still be capable of capturing PRPs and maintaining L-LTP.

1.6 The synthesis of Plasticity Related Proteins

Storage of long-term memory, or memory consolidation, requires new mRNA and protein synthesis (Davis and Squire, 1984; McGaugh, 2000). Short-term memory is insensitive to inhibitors of transcription and translation. Similarly, L-LTP depends on macromolecular synthesis while E-LTP requires neither mRNA nor protein synthesis (Kandel, 2001). Analysis of up-regulated genes after induction of LTP (Activity regulated genes (ARGs)) shows many ARGs are involved in the regulation of cell surface and adhesion, extracellular matrix, cytoskeleton, cytokine and growth factor signalling, and transcription. Also, the ARGs are localized in clusters within the chromosomes and each cluster has a regulatory molecule (i.e. CREB) that controls all the ARGs in that cluster (Cavallaro et al., 1997; Park et al., 2006). Approximately 30 min after LTP induction in the dentate gyrus of mouse, ARGs that deal with external stimuli (i.e. histocompatibility proteins, IGs) are upregulated whereas, at 60 min after induction, cytoskeleton related ARG clusters are upregulated (i.e. cell-growth, actin network, microtubules). Of these ARGs or immediate early genes (i.e. IEG is the preferred terminology particularly if the trigger to the gene upregulation is behavioural and not electrophysiological), a subset will encode for proteins with a direct effect at the synapse (i.e. PRPs, which will need to be captured by the relevant synapse). The synthesis of PRPs is required for just 1 - 2 hours around the time of induction of LTP (Nguyen et al., 1994).

There is an alternative theory to explain the need for protein synthesis for the maintenance of LTP (Routtenberg and Rekart, 2005). L-LTP is possible by post-translational modifications (PTMs) such as autophosphorylation of protein kinases, proteolytic cleavage to expose cryptic glutamate receptors, changes in cytoskeleton, translocation of receptors and kinases, and retrograde signalling. Protein synthesis is there to replenish but not to bring anything new. In accordance with this, it seems that there is a limit in the amount of mRNA being translated in the dendrite at any moment (Schuman et al., 2006). In any case, even if protein synthesis is only necessary to replenish the pool of proteins or mRNAs necessary to stabilize synaptic changes, the problem remains of how this happens and what proteins need replenishment remains.

What molecules are involved in the pathways leading to the synthesis of PRPs and what is the identity of these proteins? There is an entire subfield in cellular signalling that deals with the transmission of a signal capable of regulating gene expression (Hardingham et al., 1998; Deisseroth et al., 2003). Intracellular calcium levels seem to be the critical signal that kinases (i.e. CaMKK/IV, MAPK) sense in order to carry out the phosphorylation of transcription factors. In CA1 LTP, new protein synthesis requires activation of NMDA and D1/D5 receptors (O'Carroll and Morris, 2004; Sajikumar and Frey, 2004a). Due to the cross-tagging phenomenon explained above, we know that L-LTP and L-LTD can exhibit long-term heterosynaptic associativity between the two changes in synaptic plasticity (Sajikumar and Frey, 2004a). One possibility discussed at the end of this thesis is that the PRPs act as to "cement" the synaptic state and it locks a potentiated synapse in the same way that it locks a depotentiated one. In the 'locked synapse' hypothesis, the PRPs proteins are used to cement a change in synaptic efficacy independently of the direction of the change (LTP or LTD).

What proteins need to be synthesized or replenished in order to cement changes in synaptic plasticity? Various candidates for PRPs (and players in the biochemical pathways that lead to the availability of PRPs) have been proposed. Any of these candidates could have their function tested under a 'prp-block' experiment.

1.6.1 The pathways leading to PRP availability

The flow of intracellular information follows the ionic or second-messenger signals driven by the stimulation of receptors as they recruit intracellular pathways leading to the activation of transcription factors that will regulate the transcription of immediate early genes (Clayton, 2000). This leaves dendritic protein synthesis aside for the moment but allows us to concentrate on which signalling pathways may be necessary for the synthesis of PRPs (i.e. immediate early genes with direct effects on synaptic support and remodelling as opposed to IEGs that act as transcription factors).

Dopamine, Adenylyl cyclase, cAMP and PKA

In Aplysia cultured neurons, serotonin (5-HT) binds to receptor that activates Adenylyl cyclase, which converts ATP to cAMP. This activates the cAMP-dependent protein kinase (PKA), which recruits MAP kinase where upon both translocate to the nucleus. There, PKA activates gene expression by phosphorylating the transcription factor (CREB1) that binds to cAMP-responsive element (CRE) (Bailey et al., 2004). In Aplysia 5-HT does presynaptically what dopamine seems to do postsynaptically in hippocampal CA1 as by infusion of constitutively active PKA catalytic subunit (type Cα) into CA1 pyramidal neurons causes the same effects as 5-HT administration (Castellucci et al., 1980).

As we already know, the transcription of new molecules such as AMPAR subunits is a necessary step for the maintenance of LTP and PKA is necessary for this transcription (Nayak et al., 1998). PKA is necessary for the maintenance of 2-hour LTP (Duffy and Nguyen, 2003). Postsynaptic application of a cell-impermeant PKA inhibitor (PKI6-22) make the EPSCs decay within 1.5 h, but do not affect post-tetanic peak potentiation nor weak LTP. Application of adenylyl cyclase activator forskolin (FSK) and the cyclic nucleotide phosphodiesterase inhibitor IBMX can occlude long-lasting LTP evoked by subsequent tetanic stimulation (Frey et al., 1993; Huang and Kandel, 1994) suggesting that PKA activation is both necessary and sufficient to induce long-lasting LTP after NMDAR activation (Otmakhov et al., 2004). FSK increases the EPSC amplitude by activating predominantly postsynaptic PKA (Bolshakov et al., 1997; Duffy and Nguyen, 2003).

Rolipram, and the consequent increase in cAMP through the inhibition of phosphodiesterase type 4 (PDE4), rescues E-LTP into L-LTP and improves memory retention (Barad et al., 1998). Using rolipram, Frey has shown how PDE4 inhibition allows both E-LTP and E-LTD to be rescued into their late phases (Navakkode et al., 2004, 2005). This rescue still needs NMDAR activation, synthesis of proteins and also MAPK activation through D1/D5 receptor-mediated Rap/B-Raf pathways. The right inhibitors and stimulation protocols (i.e. PRP-bloc experiments; Fig 1.3) could also be used to confirm whether PKA activation is necessary for the synthesis of

PRPs or whether it plays a more general role as suggested by the following experiments.

While all these experiments focus on the role of PKA in the pathways leading to the synthesis of PRPs, PKA phosphorylates AMPAR subunit GluR4 at synapses and this leads to AMPAR insertion into the synaptic membrane. Also, PKA is necessary to phosphorylate AMPAR subunit GluR1 (Esteban et al., 2003). These are local actions of the kinases, and in the particular case of PKA, could mean that its actions are not only necessary for the synthesis of PRPs but also for the setting of the local tag (Nguyen and Woo, 2003).

D1 but not the D5 dopamine receptors are necessary for the synthesis of PRPs necessary for the maintenance of LTP and learning and memory (Granado et al., 2008). For dopamine receptor D1 activation, the answer to its role is already available from 'PRP-block' (Fig. 1.3) experiments that have shown their necessary role in synaptic plasticity. The D1R-driven increase in cAMP and consequent PKA activation can be restricted to the pathways leading to the availability of PRPs. This is shown by the strong-before-strong experiments (test of PRP block; Fig. 1.3) carried out by Sajikumar (Sajikumar and Frey, 2004a). Their experiments demonstrate that PKA activity after D1R activation is necessary for the synthesis of PRPs but redundant for the setting of functional tags.

CaMKK/CaMKI/CaMKIV

Parallel to the effects of D1R activation, calcium entry triggers CaMKK that, in the soma, activates CaMKIV (Bito et al., 1996; Tokumitsu et al., 2002), a nuclear kinase capable of phosphorylating Ca^{2+}/cyclic AMP-response element binding protein (CREB) (Bito et al., 1996; Chawla et al., 1998; Hardingham et al., 1998; Ho et al., 2000) and initiating the transcription of genes that synthesise the PRPs necessary for stabilizing LTP (Kang et al., 2001). Without an apparent role in the setting of the tag, this CaMKK pathway can be hypothesized to be necessary only for the availability of PRPs. The testing of this hypothesis is described in detail in chapter 7 of this thesis.

MAPK/ERK

Activation of MAPK/ERK is required for consolidation and reconsolidation of recognition memory (Kelly et al., 2003). Inhibitor UO126 blocks consolidation of object recognition memory but does not affect short-term memory. This may be because the ability of MAPK to engage the transcription of IEGs like Arc. However, after calcium entry, the MAPK pathway is modulated by CaM Kinases although CaM Kinase stimulation is not necessary for MAPK activation (Zheng et al., 2008). Interestingly, in the model used by Zheng et al., MAPK activation is not sufficient to engage Arc upregulation and needs the action of a calcium dependent pathway, probably the CaMKK investigated in this thesis. So, in addition to the direct role of CaMKIV in transcription (Enslen et al., 1994), the CaM kinase cascade mediates the activation of the MAP kinase pathway (Enslen et al., 1996).

In addition to the TrkR pathway investigated by Zheng et al. and described above, the co-activation of B-adrenergic and cholinergic receptors enhance LTP, an effect that is sensitive to MAPK inhibition (Watabe et al., 2000). In this way, MAPK integrates information from the PKA and the PKC pathways (Roberson et al., 1999). This suggests that the MAPK pathway may act as a funnel of information allowing the synthesis of PRPs. A test of 'PRP block' (Fig. 1.3), as described above, should clarify whether the MAPK pathway exerts a necessary role in the synthesis of PRPs necessary for L-LTP maintenance. A specific role in the pathway leading to the PRPs is not self-evident for MAPKs because ERKs are implicated in human mental retardation syndromes; stabilize structural changes in dendritic spines; and lower K^+ channel conductance (Sweatt, 2004). These synaptic, local effects of MAPK suggest an important role of this pathway in the setting of tags too. Tests of 'tag block' (Fig. 1.4) as well as of 'PRP block' (Fig. 1.3) should be capable of delimiting the role of MAPK.

Golgi and endoplasmic reticulum transport of PRPs

Electron microscopy shows the ER in neurons extending deep into dendritic processes (Spacek and Harris, 1997; Gardiol et al., 1999). As reviewed by Kennedy and Ehlers (2006), the ER and Golgi networks are not only necessary for the

transport and processing of PRPs but are also the primary sites of lipid biosynthesis which are necessary for maintaining dendrite size and geometry (Horton et al., 2005; Kennedy and Ehlers, 2006). There is an interesting drug, brefeldin A, that disrupts Golgi transport that, perhaps, could be used instead of anisomycin (Lippincott-Schwartz et al., 1989). Without Golgi transport, there should be no PRPs reaching anywhere while, in theory, the tag should remain in place. This is the type of hypothesis that the 'PRP-block' experiment can answer (Fig. 1.3).

Histone acetylation

Formation of long-term memory requires the regulation of gene expression after the activation of different signalling pathways. Histone-associated heterochromatin undergoes changes in structure during the formation of long-term memory. Histone acetylation has been shown to be necessary during the initial stages of consolidation of long-term association memories in a contextual fear-conditioning paradigm (Levenson et al., 2004). Activation of NMDA receptors in area CA1 *in vitro* increases acetylation of histone H3 in an ERK dependent way. Moreover, activation of ERK in area CA1 *in vitro* through either the protein kinase C or protein kinase A pathways, also increases histone H3 acetylation. Elevating levels of histone acetylation enhances induction of long term potentiation at Schaffer-collateral synapses in area CA1 of the hippocampus, a candidate mechanism contributing to long term memory formation *in vivo* (Levenson et al., 2004). It should also be noted that acetylation of histones can in some cases be self-perpetuating, which during development, may serve as a long term cellular memory, creating a functionally stable chromatin state and thus chronic changes in the rates of specific gene expression (Turner, 2002). Histone acetylation seems an excellent candidate for a molecule with a necessary role in the process capable of affecting the cell-wide spread of PRPs.

Transcription factors

Transcription factors are proteins that regulate the transcription of certain genes. Because their actions are nuclear and not synaptic, they do not qualify themselves as plasticity related proteins that will be capture by tagged synapses. Transcription factors, nonetheless, are candidates to have a critical role in making the PRPs available. The actions of protein synthesis inhibitors show the need for the immediate synthesis of some proteins right after the induction of synaptic plasticity. There is then an early genetic response to synaptic inputs that relies on transcription factors to engage the transcription of late response genes.

Zif268

The early response proteins consist of transcription factors like Zif268, which engage the transcription of other genes coding for the PRPs themselves (see below). For example, Zif268 interacts with CREB binding protein and others to regulate the expression of synapsin 1 (Thiel et al., 1994; Silverman et al., 1998). Synapsin could well act as a PRP in this model and its availability would therefore be dependent on the successful production and activation of transcription factors.

CREB

CREB-mediated transcription correlates with associative memory formation in the HPC (Tully, 1998). One pathway leading to CREB phosphorylation involves adenylyl cyclase activity after dopaminergic, noradrenergic or even serotonergic input. The increased levels of cAMP activate PKA that together with MAPK can translocate to the nucleus where they activate transcription factors that bind to the cAMP-responsive element (CRE), as well as CRE binding protein 1 (CREB1) (Bailey et al., 2004). Another pathway leading into CREB phosphorylation involves the CaMK pathway as explained above (Bito et al., 1996; Ho et al., 2000).

Phosphatases like calcineurin also contribute to CRE-dependent transcription while blocking serum response element (SRE) transcription (Lam et al., 2009).

All this convergent pathways into CREB suggest that the CREB regulatory unit may serve to integrate signals from various transduction pathways. In mice with truncated CBP there are memory deficits (Wood et al., 2005) and this opens the possibility of a necessary role of CREB transcription in the synthesis of the PRPs involved in synaptic plasticity. CREB becomes then a candidate for 'PRP-block' experiments.

1.6.2 The molecular identity of PRPs

The concept of plasticity related proteins (PRPs) in the context of synaptic tagging and capture refers to products of immediate early genes with a direct effect at the synapse. This definition is different and more restrictive than that of activity regulated genes (ARGs) or immediate early genes (IEGs). Within the STC framework, PRPs are captured by synaptic tags and therefore are a subgroup of ARGs or IEGs that also include transcription factors and modulators like Zif268. The requirement for the effect of PRPs to take place at the synapse leaves out all IEGs with actions in neurosecretion or intracellular signalling.

PKC-PKMζ

PKMζ is a persistently active PKC isoform that acts by increasing the surface expression of AMPAR and consequently enhancing synaptic transmission (Ling et al., 2006).

No persistent increase of PKMζ follows STP but it does after LTP induction. Anisomycin and cycloheximide block the PKMζ increase and LTP at the same time (Osten et al., 1996). The autonomously active isoform of PKC is necessary and sufficient for maintaining LTP (Ling et al., 2002). An inhibitor of PKMζ reverses L-LTP but not L-LTD and this suggests that PKMζ is a PRP only necessary for L-LTP. Activation of PKC induces rapid morphological actin-based plasticity in dendrites of

HPC neurons (Pilpel and Segal, 2004). PKMζ could be the first LTP specific PRP to have been identified (Sajikumar et al., 2005c).

A key experiment explains that PKMζ lacks a regulatory domain like PKC and is then constitutively active once synthesized from mRNA (Pastalkova et al., 2006). This study used a cell permeable regulatory subunit that binds to PKMζ to reverse L-LTP 22 hours after induction in CA1 *in vivo*. They also show that the same injection 22 hours after training decreases the performance in a place avoidance task.

Inhibition of PKMζ did not affect baseline AMPA receptor-mediated synaptic transmission or an early phase of LTP. In contrast, the inhibitor reversed established LTP when applied 1, 3, or 5 h after tetanic stimulation (Serrano et al., 2005).

The ability of PKMζ inhibitors to affect all potentiated synapses complicates their use in PRP-block and tag-block experiments.

SNAP-25

As a synaptosomal protein, SNAP-25 plays a role in a number of neuronal functions including axonal growth, dendrite formation, fusion of synaptic vesicles with membrane and the expression of long-term potentiation (LTP) in the hippocampus. SNAP-25 has been identified as one of the differentially expressed genes in the hippocampus upon behavioural training. The inhibition of SNAP-25 with intracerebroventricular antisense oligonucleotide caused a deficit in long- but not short-term memory for step-down inhibitory avoidance. Intra-CA1 infusion of the SNAP-25 antisense oligonucleotide impaired long-term contextual fear memory and spatial memory and interfered with the LTP of synaptic transmission in the CA1 region. The inhibitory effect on LTP was not mediated by a presynaptic mechanism because paired pulse facilitation of synaptic transmission was not affected after administration of the antisense oligonucleotide. Together, the results suggest that SNAP-25 in the CA1 region is involved in memory consolidation (Hou et al., 2004). And this gives SNAP-25 a postsynaptic role (maybe bringing NMDA receptors to the synapse).

Arc

Arc/Arg3.1 binds the endocytic proteins dynamin-2 and endophilin-3, forming a complex that regulates the endocytic trafficking of AMPA-type glutamate receptors

(Shepherd et al., 2006). Lack of Arc leaves AMPAR in the membrane surface (Chowdhury et al., 2006) and this suggests that the function of Arc may be to down-regulate AMPAR expression at non-potentiated synapses (Abraham and Williams, 2008).

One-trial memory, just one run around a new track, recruits IEG expression, including Arc (Miyashita et al., 2009) and b-adrenoreceptor agonists in the basolateral amygdala do increase the levels of Arc protein in the HPC and the memory in an inhibitory avoidance (IA) task 48 hour later (McIntyre et al., 2005). The increase in Arc is translational and not transcriptional since there is no increase in mRNA levels, only increase in protein levels. In the dentate gyrus, to target Arc to stimulated synapses, NMDARs need to have been activated (Steward and Worley, 2001). Similarly in CA1 pyramidal cells, Arc protein levels increase after NMDAR activation as well as G-coupled receptor activation of PKA (through dopamine and noradrenaline) (Bloomer et al., 2008).

The role of Arc as a PRP seems unknown or at least controversial (Tzingounis and Nicoll, 2006). I will suggest in the discussion to this thesis a role of Arc in LTD expression and maintenance (Chapter 11).

Homer

Homer 1a is a PRP upregulated after NMDAR stimulation (Sato et al., 2001) and the induction of long-term potentiation (Kato et al., 1997). Homer1 links to postsynaptic density proteins (Tu et al., 1999; Xiao et al., 2000). Homer 1a has recently been shown to behave precisely according to the STC hypothesis in that it is distributed all along the dendrites but does not enter the dendritic spines unless there is NMDAR activation (Okada et al., 2009).

Homer, and most of the molecular players described above will have critical roles in the model of synaptic plasticity and synaptic tagging described in chapter 11.

1.7 Synaptic plasticity and memory

So far, I have presented a review of the literature revolving around synaptic plasticity in neurons. The main reason behind so much interest in the mechanisms involved in synaptic plasticity is that these changes are the preferred physiological model for the formation and maintenance of memories (Matthies, 1989a). The synaptic plasticity and memory hypothesis states that …

"Activity-dependent synaptic plasticity is induced at appropriate synapses during memory formation, and is both necessary and sufficient for the information storage underlying the type of memory mediated by the brain area in which that plasticity is observed."

(Morris et al., 2003).

The hypothesis predicts four testable conditions (Martin and Morris, 2002b) and evidence for and against these predictions is discussed below:

Detectability: If an animal displays memory of some previous experience, a change in synaptic efficacy should be detectable somewhere in its nervous system. This type of correlation is technically difficult to obtain specially electrophysiologically where even the changes in temperature after motor activity can alter the responses recorded via electrodes (Moser et al., 1993). With improved techniques and proper controls, however, a critical experiment in supporting this prediction showed that after inhibitory avoidance a few populations of cells in CA1 show potentiation (Whitlock et al., 2006). More recently, the phosphorylation of AMPAR subunit GluR1 at the Ser 831, known to be involved in LTP, has also been associated to memory formation (Shukla et al., 2007).

Mimicry: Changes in synaptic strength should be sufficient to encode a memory. This is the most difficult prediction to assess experimentally. It requires the production of controlled changes in synaptic efficacy and as a result, the construction of measurable memory for something never experienced through external sensation (false memory).

Anterograde alteration: Interventions that prevent or limit the induction of synaptic weight changes during a learning experience should block or impair the animal's memory of that experience.

For instance, NMDA receptor antagonists (AP5) applied during encoding, impair memory performance (Morris et al., 1986). How the original studies in the water maze fit the prediction of anterograde alteration is still controversial since pretraining in the spatial task prevented the effect of AP5 (Bannerman et al., 1995). Nevertheless, more modern studies using improved controls and genetic techniques keep showing the need for NMDAR activation in the hippocampus for one-trial learning in the water maze (Nakazawa et al., 2003).

There are other correlational studies that show interference with LTP by blocking mGluRs (Manahan-Vaughan and Reymann, 1995) correlates with impaired spatial learning (Holscher et al., 1996) More recently, targeted destruction of only those cells undergoing PRP upregulation (through CREB activation) in the amygdala, prevented the encoding of fear memory (Han et al., 2009).

Retrograde alteration: Interfering with changes in synaptic efficacy after a memory has been encoded should affect the memory too. Inhibiting PKMζ, which we know disrupts the maintenance of LTP, after a memory for a spatial location has been encoded, disrupts that memory (Pastalkova et al., 2006).

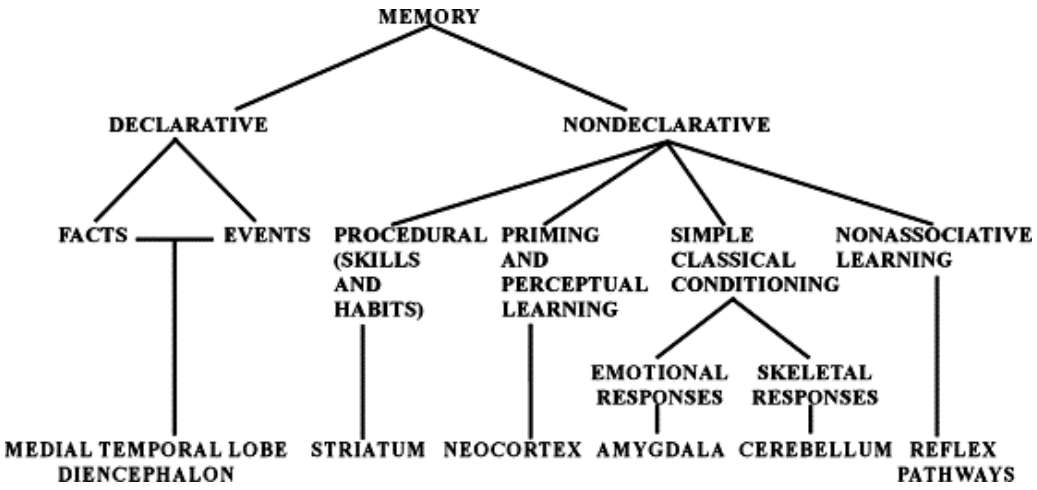
With the exception of the extremely complex condition of mimicry, the synaptic plasticity and memory hypothesis has been validated by enough results so as to shift the interest and efforts of neuroscientists from the question of "whether" to "how" does synaptic plasticity account for learned behaviour. Most research on the mechanisms of synaptic plasticity has been done on the hippocampus at the expense of other brain structures (neocortex, amygdala, cerebellum, …). For instance, the heterosynaptic plasticity explained by the STC hypothesis has only been reported in the hippocampus. Why is the hippocampus the focus of so much research on memory and its mechanisms?

1.7.1 Brain circuitry involved in memory

Memory is defined in psychology as the ability to acquire, store and recall information. Independently of the different types of memories and brains structures contributing to them, there are three requirements for a memory to be able to affect the behaviour of an organism. A memory needs to be encoded, stored and retrieved. Encoding requires the registration and the processing of information. The storage stage defines all those processes that lead to a more or less permanent record of that information. Finally, retrieval or recall is the process whereby the memory is called back and allowed to influence the behaviour of the organism. Every memory system shares these three stages and requirements. Figure 1.5 describes a widely accepted division of memory systems with the corresponding brain structures sustaining them (Squire, 2004).

Fig. 1.5 A taxonomy of mammalian long-term memory systems.

The taxonomy lists the brain structures thought to be especially important for each form of declarative and nondeclarative memory. In addition to its central role in emotional learning, the amygdala is able to modulate the strength of both declarative and nondeclarative memory. From Squire 2004 (Squire, 2004).



1.7.2 Role of the hippocampus in memory

In 1953 patient Henry Gustav Molaison underwent a treatment for epilepsy that removed his hippocampi and other regions of the temporal lobe. The memory deficits that he showed since underlined the importance of the temporal lobe and the hippocampus in memory formation and focused memory research on the hippocampal formation for decades to come. Other cases like that of R.B. who suffered hypoxia-induced loss of pyramidal neurons in area CA1 (Zola-Morgan et al., 1986) and others (Rempel-Clower et al., 1996) added to the importance of the hippocampus in human memory.

The hippocampus is a forebrain structure that lies within the corpus callosum and stretches caudally from the septum, and latero-ventrally around the thalamus (Amaral, 2004). The hippocampal formation consists of the hippocampus proper |(i.e. Ammon's horn), the dentate gyrus, the subicular complex, and the entorhinal cortex. The hippocampus proper is divided into three subfields on the basis of the size of the cells and their distribution. These regions are CA1 (Cornus Ammonis 1), CA2 and CA3. Pyramidal cells in CA3 receive a prominent mossy fibre input from the dentate gyrus. Then these CA3 cells innervate the CA1 region, which itself also receives input from the entorhinal cortex. CA3 (recurrent network) seems to act as an autoassociative memory network supporting the dynamic competition between pattern separation and pattern completion processes, while CA1 (feedforward network) reacts with small changes in network activity to small changes in the environment (Leutgeb et al., 2004).

There are multiple memory systems with distinct anatomical organizations and functions. Declarative memory involves remembering facts and events (medial temporal lobe). Procedural memory involves the acquisition of skills and other forms of non-conscious learning (basal ganglia and cerebellum) (Squire, 1992). The role of the hippocampus in memory formation has been debated extensively and the different models proposed are of relevance to this thesis because of the potential of the STC hypothesis to further our understanding of hippocampal function.

Most models of the neurobiology of memory have been based on the idea that information is stored as distributed patterns of altered synaptic weights in neuronal networks. The discovery of place cells in freely moving rats (O'Keefe and Dostrovsky, 1971) helped to undermine stimulus-response theories that drove psychology in the first half of the twentieth century. The hippocampus was proposed to maintain a cognitive map, in which the activity of place cells represents where the animal is in the form of a map (Wagatsuma and Yamaguchi, 2007). In addition to the cognitive map, Eichenbaum proposed that the hippocampus is not restricted to spatial processing but that it also represents sequences of events that compose episodic memories (Eichenbaum et al., 1999). This representational flexibility is held to be relational with the hippocampal network enabling logical inferences to be made about events or items that may never have been experienced in the past. This representational flexibility allows for previously learned information to be applied in novel situations involving different actions. The hippocampus acts as an 'associator' of different items and events, and the resulting network of information is not rigidly tied with a specific task but can be accessed and used to support a multitude of goals. This is supported by water maze impairment of hippocampal-lesioned rats when changing the start point (Eichenbaum et al., 1990).

The STC hypothesis and the phenomenon that it describes allow for the testing of interesting predictions of models based on Hebbian learning rules. The interactions between the tag and the PRP bring spatial and time constraints to the heterosynaptic modulation of memory encoding in pyramidal cells. Following the STC hypothesis, as long as similar cell populations are used to encode two different stimuli, according to the cognitive map model (O'Keefe and Dostrovsky, 1971), the encoding of one spatial memory might sometimes be capable of influencing the persistence of a second spatial memory. According to the representational flexibility model of hippocampal function (Eichenbaum et al., 1999), spatial as well as non-spatial associative memories should be also able to interact and benefit from the heterosynaptic modulation described by the STC hypothesis. On the other hand, the sparse hippocampal associations of the hippocampus of association models of hippocampal function (Rudy and Sutherland, 1995; O'Reilly and Rudy, 2000) should make the electrophysiological interactions described by the STC hypothesis very

difficult to detect in hippocampal dependent memories and behaviour. Later in the thesis, I will present evidence of how two independent experiences can interact in a manner analogous to that described for two independent synaptic pathways in the STC hypothesis. For these experiments to translate the hippocampal phenomenon of STC into its behavioural correlate I needed a hippocampal dependant task that allowed the manipulation of a weak, short-lasting memory.

The experiments described in chapters 8 to 10 benefit from the relative weakness and ephemerality of the memory encoded in one-trial match to place tasks as developed in the Morris water maze (Steele and Morris, 1999) and in a dry maze 'event arena' (da Silva and Bast, 2004). What sort of neuromodulatory manipulation will stabilize the maintenance of the 'weak' memory by making PRPs available to the synapses encoding it?

1.7.3 Neuromodulation of hippocampal memory

The STC hypothesis furthers our understanding of heterosynaptic modulation of synaptic plasticity, this is, how independent inputs can interact at their cellular targets to strengthen synaptic changes. Methodologically, electrical stimulation of the hippocampal slice elicits a response that can be measured by monitoring the movement of ions into the target cells (EPSPs). However, an electrical recording does not monitor the activation of metabotropic receptors or the subsequent activation of intracellular second messengers. In spite of this, we know that high-frequency stimulation will not only recruit glutamatergic axons but also axons from neuromodulatory cells (dopamine, acetylcholine, serotonin, …). These axons are sectioned during the preparation of acute slices but their terminals remain functional and are recruited by the HFS stimulation typically used to induce synaptic plasticity.

It is of relevance then to investigate what is the importance of these inputs both in synaptic plasticity and in hippocampal memory. I focus on dopamine because the experiments described in this thesis make us of dopamine receptor blockers, but the hippocampus is also influenced by adrenergic, serotonergic and cholinergic neuromodulatory inputs.

The dopaminergic system in the maintenance of memory and synaptic plasticity

Dopamine has distinct actions in different areas of the brain. Endogenous dopamine, which depends on the activity patterns of dopamine midbrain neurons in freely moving animals, appears as a key regulator in specific synaptic changes observed at certain stages of learning and memory and of synaptic plasticity (Jay, 2003).

Dopamine is one of the most important modulatory input involved in heterosynaptic plasticity in the hippocampus. In the CA1 region of the HPC, dopaminergic antagonists block the late-phase of LTP (Frey et al., 1990; O'Carroll and Morris, 2004). There is no dopamine release during baseline stimulation at 0.33Hz but there is release of dopamine at 100Hz. The impact of dopaminergic input can be inhibited by the compound SCH23390 which blocks the D1 receptor (Frey et al., 1991). The consequent lack of dopaminergic action disrupts the maintenance of synaptic plasticity (O'Carroll and Morris, 2004) as well as the maintenance of memory (O'Carroll et al., 2006). Initially, D1R agonists were thought to induce late-LTP through coupling with adenylyl cyclase without the need for electrical stimulation (Huang and Kandel, 1995). These results would make dopamine an initiator of synaptic plasticity rather than a modulator. This is controversial, as it would imply that D1/D5R activation is capable, on its own, to set local tags and engage the synthesis of PRPs. However, other laboratories that activated D1/D5 receptors with agonists obtained a cAMP increase but not a potentiation of EPSPs if they only used constant baseline stimulation (Swanson-Park et al., 1999; Mockett et al., 2004). Other experiments clarifying the role of dopamine on CA1 synaptic plasticity have shown that the effects of dopamine agonists require constant test stimulation and NMDAR activation (Navakkode et al., 2007). This indicates that dopamine release is insufficient to induce LTP unless there is co-ocurrent NMDAR-dependent activity at glutamatergic synapses. These results fit with the model of heterosynaptic interactions proposed by the STC hypothesis since the synergistic interactions between NMDAR and D1R could account for the setting of tags and the subsequent availability of PRPs, respectively. Indeed, downstream of D1R and

adenylyl cyclase, PKA activation is necessary L-LTP and capable of inducing a certain form of protein synthesis dependent LTP (Frey et al., 1993).

The role of dopamine in the STC model can be assessed by combining tetanizations of independent inputs with or without the drug being present (explained above section 1.3). Briefly, if strong stimulation (leading to the induction of LTD or LTP) is delivered to one set of synapses under the effects of the D1/D5R blocker SCH 23390, the change in synaptic efficacy fails to be maintained for longer than 4 hours (no L-LTP). However, before the drug is added to the preparation, if another independent but convergent input has previously experienced strong stimulation, both sets of synapses show L-LTP. Something necessary for the maintenance of L-LTP is missing in the first experiment that the additional strong stimulation provides in the second experiment. This necessary element is capable of acting on synapses independent from those that activated it. These results with D1/D5R blockers parallel those with anisomycin and suggest that dopaminergic inputs into the CA1 region of the hippocampus are necessary to make PRPs available for the stabilization of synaptic changes. Using these elegant experiments, D1/D5R block is shown to impair the synthesis of PRPs but does not have a deleterious effect on the setting of the tag (Sajikumar and Frey, 2004a). In other words, dopamine is necessary for the availability of PRPs but is not necessary for the setting of tags. This is an example of a PRP-block experiment (Fig. 1.3).

With some knowledge of the role of dopamine in synaptic plasticity, the question becomes that of translating that knowledge into behaviour (i.e. the encoding, expression and maintenance of memories). What can the STC hypothesis, and the role of dopamine within it, tell us about memories?

1.7.4 The STC hypothesis relevance to the link between synaptic plasticity and memory.

Besides the aforementioned uses of synaptic tagging as a tool to elucidate the roles of distinct molecules in synaptic plasticity, it is also of interest has to ask whether the heterosynaptic plasticity behind the STC hypothesis described in hippocampal slices has any behavioural relevance. The capture of PRPs (GluR1 subunit of the AMPAR) has been tracked to specific types of synapses after learning (Matsuo et al., 2008) suggesting that the mechanisms of tagging and capture studied *in vitro* may have a role to play *in vivo*. Which of the properties of CA1 pyramidal cells responsible for the heterosynaptic plasticity described in the STC hypothesis are of relevance to learning and memory?

The synaptic tagging and capture hypothesis implies a potential dissociation between the induction and tagging of LTP and the *de novo* protein synthesis required to stabilise it. A behavioural analogue to the STC model would be one in which an event stimulating proteins synthesis could provide the plasticity proteins that synaptic tags capture to stabilise the persistence of memory traces. The tags could be set by separate events in the common pool of neurons. This translation of the STC hypothesis into behaviour is called 'behavioural tagging'. It predicts that an encoding event that normally produces a short-lasting memory, when experienced around the time of another event capable of producing a long-lasting memory, would now itself produce a long-lasting memory.

Although not restricted to such situations, flashbulb memories are a case in point. The term 'Flashbulb memory' refers to the vivid memories that seem to be encoded after a behaviourally surprising or emotional event (Brown and Kulik, 1977). Importantly, these memories are not restricted to the relevant event but extend to less important facts or situations that happened around that time. Examples of these vivid memories are the September 11, 2001 attacks in the USA and all the detailed information that subjects can recall about their personal experience at that same time. There is discussion as to whether the mechanisms that allow for such vivid memories are acting at the time of encoding or whether this phenomenon is an artefact of repeated reminding and rehearsing of the memory (Talarico and Rubin, 2003).

There is however evidence that surprising experiences improve memory performance for unrelated items. Human subjects remember lists of terms better if learned around the time of exposure to a novel and surprising event (Kock et al., 2008). The surprise (e.g. picture of snake) affects the memorization of unrelated terms presented both before and after. Can this observation be accounted for by the mechanisms behind the STC hypothesis? What does memory consolidation need and to what extent do these requirements follow those of synaptic plasticity? In the same way that synapses can be temporarily tagged and then capture PRPs induced by an independent event, and so consolidate that synaptic change, can weak memories that usually decay after a short time interval, benefit from PRPs brought about by an independent behavioural event? How closely would the mechanisms of memory modulation follow those behind the STC hypothesis?

In chapters 8, 9 and 10 I will describe a series of experiments that allow for the investigation of behavioural tagging. The experiments involve the use of a weak or decaying memory as well as a behavioural event capable of upregulating PRPs in the same cells encoding for the weak memory, without disrupting the original memory. We decided to work on hippocampal dependent tasks (i.e. one-trial memory in the water maze and in the event arena) and the biggest challenge was to develop the behavioural experience that would modulate a weak memory via the *de novo* synthesis of PRPs. We focused on novelty and stress for the following reasons:

Novelty

Novel experiences can affect the maintenance of LTP (Kemp and Manahan-Vaughan, 2004). In this way, CA1 LTP can be facilitated by exploration of an empty novel environment and depotentiated by object exploration. Novelty exploration releases dopamine in the hippocampus (Ihalainen et al., 1999), and this dopamine lowers the threshold for the induction of weak LTP (Li et al., 2003) and this effect is dependent on activation of D1/D5 receptors in the area CA1 of the hippocampus.

The neuromodulators responsible for this effect are under discussion. Exposure to novelty activates mesolimbic dopaminergic neurons, which innervate several brain areas including the hippocampus (Schultz, 2000). Novelty exposure is also associated with increased cholinergic (Acquas et al., 1996) and noradrenergic

(Vankov et al., 1995; Kitchigina et al., 1997) drive to the hippocampus, and activation of muscarinic cholinoceptors and B-adrenoceptors can facilitate LTP induction. However, Li et al. (2003) injected selective B-adrenoceptor antagonist propanolol 10 min before novelty exposure and this did not block facilitation of LTP induction (Li et al., 2003). This means that the co-ocurrent activation of noradrenergic receptors is not necessary for this facilitation of LTP by novelty. A similar result was obtained with scopolamine, a muscarinic receptor antagonist. However, against the lack of effect of propanolol in LTP, there is a study in humans that shows a facilitatory effect of adrenergic input in emotional memories in humans (Cahill et al., 1994).

Concerning the role of dopamine in LTP and memory, hippocampal dopamine depletion impairs (and D1 agonists enhance) certain types of hippocampus dependent learning: spatial memory in water maze (Gasbarri et al., 1996), spatial memory in water maze for aged-impaired rat (Hersi et al., 1995) and mice (Bach et al., 1999). Other studies report that novelty exploration:

- Promotes the reversal of previously established LTP (depotentiation) *in vivo* (Xu et al., 1998).
- Rescues STD into LTD in Lister Hooded rats and enhances LTD in Wistar rats (Manahan-Vaughan and Braunewell, 1999).
- Reverses 14 day long LTP but not 90 day long LTP (Abraham et al., 2002).

What this tells us is that novelty exploration weakens recently strengthened synapses (Xu et al., 1998), but also strengthens sparsely distributed networks. More importantly to my thesis, when novelty has an effect it is a facilitation in induction and reversal in expression. Stimulation of the perforant path *in vivo* 15-30 min (but not 1 h.) after exploration of a novel environment (for 15 min) rescued E-LTP into L-LTP (Straube et al., 2003). Application of B-adrenergic antagonist propanolol (30 min before tetanus) or anisomycin (60 min before tetanus) prevented the LTP reinforcement.

Novelty was used in chapters 8 and 9 of this thesis to modulate the maintenance of a short-lasting spatial memory.

Stress

Swimming in colder water (19 degrees C) improved memory performance when compared to swimming in warm water (25 degrees) in the watermaze (Akirav et al., 2004). Also, cold stress elevates corticosterone levels but does not enhance nor impair LTP (Bramham et al., 1998). From these observations, we predicted that acute cold stress could produce the PRPs necessary to maintain a weak memory without any adverse effect and tested this prediction in the experiments described in chapter 10 of this thesis.

However, Diamond and Woodson show that watermaze platform memory is clearly impaired after 30 min if combined with a stressful event (electric shock) (Diamond et al., 2004). The authors claim that the impairment may be due to the new stressful memory being encoded and disturbing the distribution of synaptic weights that would encode the platform location.

Electrophysiological experiments show that behavioural stress facilitates the induction of long-term depression (LTD) in hippocampus (Xu et al., 1997; Chaouloff et al., 2007). It does so by acting on NR2B containing NMDARs and without using NR2A or synaptic NMDARs (Yang et al., 2005). This suggests that LTD observed in slices from stressed animals is triggered primarily by extrasynaptic NMDAR activation. Yang et al. (2005) also finds that the facilitation of LTD in stressed rats is due to the blockade of glutamate uptake. The risk of using stress as a behavioural manipulation can be summarized as follows: Behavioural stress blocks glutamate re-uptake, therefore enhancing LTD, which can be excitotoxic in nature. The consequent modifications in synaptic weights would interfere with the correct encoding or maintenance of relevant memories. Despite the fact that stress could induce synaptic depression, the cold water manipulation was still worth using as a PRP-inducing protocol since from the STC literature we know that it does not matter whether the upregulation of PRPs is triggered by the induction of potentiation or depression mechanism, weakly tagged synapses are capable of capturing the PRPs (cross-tagging (Sajikumar and Frey, 2004a)). Also, it is worth remembering that LTD could serve as a mechanism for memory storage as well as LTP (Bear and Abraham, 1996; Kemp and Manahan-Vaughan, 2004). Because of this potential role

of LTD in the encoding of memories, the electrophysiological results reported here support the prediction of stress engaging plasticity mechanisms. Cold-water stress was used in this thesis (Chapter 10) in an attempt to upregulate the PRPs necessary to stabilize a weak memory thereby lengthening its persistence.

1.8 The plan for the present experiments

The focus of the present experiments is two-fold. First, electrophysiological experiments will try to learn more about the signal-transduction pathways involved in the setting of the tag and those necessary for the synthesis of PRPs. I will use PRP-blocking as well as the tag-blocking protocols as described above (Fig. 1.3 & 1.4) to test necessary roles in the pathways leading to synaptic plasticity for two CaM-kinases. CaMKII is predicted to have a limited role locally at the synapse, which is predicted to be selectively revealed by the tag-block protocol. On the other hand, the inhibition of CaMKK should only block the synthesis of PRPs. To test this dissociation, parallel but different series of experiments will be conducted (PRP-block protocol).

Secondly, I will try to translate the STC hypothesis into a behavioural correlate. This will be a test of the predictive value of the STC hypothesis together with the synaptic plasticity and memory hypothesis (Martin and Morris, 2002a). Weak memories will be paired with PRP upregulating events with the prediction that with PRPs present in the cells encoding the memory, the weak memory should be maintained for longer. We used novel exploration in chapters 8 and 9 and cold water stress in chapter 10.

From the electrophysiology results, an updated version of the STC hypothesis will be proposed in chapter 11.

Chapter 2. Materials & Methods

2.1 Electrophysiology of the late phases of long term potentiation

Acute brain slices allow for the investigation of nervous tissue at different stages of the animal's life (Deupree et al., 1991) and can be used to study the connectivity of neurological tissue at around the time of extraction. The health of the preparation and the stability of the recordings are the main challenges of acute slice electrophysiology both for interface (Skrede and Westgaard, 1971; Haas et al., 1979) and submerged setups (Nicoll and Alger, 1981). There are ways of maintaining submerged slice preparations viable for more than ten hours (Reymann et al., 1985; Fonseca et al., 2004). However, submerged slices do not recover from periods of anoxia in the same way as interface slices (Croning and Haddad, 1998). In submerged slices, the effects of lack of oxygenation can be expressed even after 6 h after the period of hypoxia (Watson et al., 1994). Submerged slices quickly suffer from glycogen depletion, increased coated vesicles and microtubule disassembly (Fiala et al., 2003) and the extracellular pH is also more sensitive to changes in temperature than in interface setups (Schuchmann et al., 2002). There are detailed comparisons of the differences between submerged and interface chamber, as well as thorough reviews of the variables relevant to slice electrophysiology (Skrede and Westgaard, 1971; Reid et al., 1988; Aitken et al., 1995; Lipton et al., 1995).

For the reasons mentioned above I have opted to work in an interface setup. Still, with this technology, long-term electrophysiology is hindered by the sensitivity of the exposed surface to small changes in temperature and humidity. Here I describe how to maintain electrophysiological stability for periods longer than 10 hours by controlling the temperature and humidity levels on the whole electrophysiology rig and not only the slice chamber. The stability achieved through the methodology described here was fundamental in answering the questions presented in chapters 3 to 7.

2.1.1 The slice preparation

Artificial cerebrospinal fluid (aCSF) was prepared with the following concentrations: NaCl 124 mM, KCl 3.7 mM, KH_2PO_4 1.2 mM, $MgSO_4(7H_20)$ 1.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.6 mM, D-glucose 10 mM (pH = 7.4). Some laboratories find it useful to increase the aCSF levels of potassium to around 5 mM in order to obtain measurable signals. Our experiments were run closer to the normal *in vivo* K^+ level of 3 mM (Mayevsky et al., 1974). aCSF was prepared every morning fresh in millipored distilled water. 200 ml were reserved for the brain dissection and placed on a -20 °C freezer for 1 h. The remaining aCSF was divided between three rigs and after circulation it was discarded. The aCSF was not re-circulated.

Slow perfusion rates (<1 ml/min) were critical and 0.4 ml/min was used as the standard flow rate. Running at such slow flow rates may carry the problems of a long delay when applying drugs and a low turnover in the dead volume once in the slice chamber. The delay between the time at which the drug starts flowing and the time it reaches the slice (transit time) was minimized by using tubing with small inner diameter (Cole-Parmer Tygon Tubing ID: 1.14 mm; OD: 2.87mm). The transit time was about seven minutes and was taken into account when infusing drugs. The hippocampal slices rested on top of 8 layers of lens tissue paper (Whatman 105) placed on top of the hard surface of the slice chamber (BSC2 Scientific Systems Design). This ensured the tissue was well soaked in the lens tissue paper while its surface remained exposed to air. All the aCSF volume held in the chamber was contained in the 2 cm wide by 2.5 cm long layers of lens tissue paper and amounted to 0.475 ml (i.e. dead volume in the chamber). All the experiments were run with the same low flow rate (0.4 ml/min) to minimize mechanical disruption of the recordings. Under these conditions, the full recycling of the aCSF every 71 seconds translates into 16.9 washes in 20 minutes. Even though every drug has its own characteristics (i.e. site of action, affinity), we predicted that 20 minutes and more than 15 washes would be enough to wash out the drugs used in these experiments and our results suggest so. Whenever drug wash-outs could be critical, alternative experiments were used to corroborate the interpretation of the result.

As the experiments run overnight, the one hour wait until the 200 ml of aCSF reserved for dissection becomes ice-cold was invested in stopping the previous day experiment as well as washing the tubing and the chamber with 3% H_20_2 for 20 min and then with distilled H_20 before starting with the freshly prepared aCSF.

The original design of the BSC2 chamber was modified by only using one inflow and one outflow channel (Figure 2.1). The flow was controlled via a Watson Marlow pump running one cartridge as the inflow and two cartridges as the outflow (Watson Marlow 205S with PVC manifold tubing ID: 0.8mm and OD: 1.42mm). The excess suction kept the aCSF levels constant. The 2x3 cm 8-layered lens tissue paper (Whatman 105; 8 layered 2x3 cm rectangle) was placed on top of the chamber, at mid distance between the inlet and outlet. Then, freshly chlorided electrodes (made with silver wire (Advent: Silver wire purity 99.99%, ∅: 0.37mm) soldered to a gold pin) were positioned as in Figure 2.1. The use of monopolar stimulation requires a reference electrode and a return electrode in contact with the aCSF. The reference electrode was placed into the inflow hole at the top of the chamber and the lens tissue paper was covered with the wide rhomboid insert provided with the BSC2 chamber. Once the insert was in position, the return electrode was placed into the outflow hole at the bottom of the chamber.

At this stage, the chamber was ready to receive the brain slices. A 7 to 8 week old male Wistar rat was anaesthetized with halothane or isofluorane, before the brain was removed and sectioned as described previously (Leutgeb et al., 2003). 400μm thick brain slices were sectioned with a Vibratome (Campden Instruments Integraslice 7550 PSDS) using stainless steel blades (Campdem Instruments 7550/1/SS). The brain slices were kept in a resting chamber with oxygenated aCSF for less than 5 minutes before being transferred into the experimental chamber. Slices were moved by gentle suction into a wide pipette and were never in contact with a brush or any other hard surface during these movements. Two brain slices were placed in every chamber by stopping the aCSF flow, flooding the chamber with 2 ml of aCSF and helping the slices float into position, perpendicular to the direction of aCSF flow, one downstream from the other (Figure 2.1).

2.1.2 Electrophysiology

Five minutes after the transfer of the slices into the recording chamber, the three monopolar stainless steel stimulating electrodes (A-M systems) and the one stainless steel recording electrode were positioned as in Figure 2.1. The electrodes were modified at least 24 h before the experiment by threading them through 5 cm long glass capillary (Harvard Apparatus GC200-10 broken in half) and glued with clear weld epoxy glue (Top Stik®). Once the electrodes were in position, the front screen of the rigs was lowered and the slices were allowed to rest for at least two hours before any type of recording started. The importance of this resting period has been described before (Sajikumar et al., 2005a) and has been shown to be necessary to allow kinase phosphorylation levels to fall to basal levels or to reach a steady state (Ho et al., 2004).

After every experiment, the stimulating and the recording electrodes were cleaned with 3% H_2O_2 for at least one hour and rinsed with aCSF before placing them on a new slice. They were reused for as long as the tips remained sharp. Test stimulation was 0.0067 Hz, 1 pulse per 150 s, resulting in one of the 3 channels being stimulated every 50 s (0.02 Hz). The rate was chosen to satisfy the criterion for low stimulation rate that would let activity rates of kinases and other molecules drop to a resting state between stimulation (Sajikumar et al., 2005a). At this low rate of stimulation, hippocampal slices react more slowly to drugs like Anisomycin than at faster rates (Fonseca et al., 2006a; Sajikumar et al., 2008). Also, slow stimulation increases the viability of the slice (Schurr et al., 1986). Critically, test stimulation activates NMDAR and the molecular cascades linked to it (Navakkode et al., 2007). Minimizing the impact of test stimulation is in the interest of stability as well as reproducibility of the data from experiment to experiment.

The absolute level of the field-potentials showed slope functions of -0.25 ± 0.09 mV/ms across all 157 pathways monitored in *stratum radiatum* and + 0.20 ± 0.01 mV/ms across the 92 pathways monitored in *stratum oriens*.

2.1.3 Whole rig temperature control

The rigs of different sizes (H x W x D (cm): 180 x 100 x 85; 180 x 110 x 90; 165 x 115 x 95) were thermally isolated by wrapping them in a double layer of aluminium foil. Heat was provided by 1 kW heaters placed right underneath the rigs. The current temperature was sensed via a temperature probe (Type K thermocouple) positioned half a centimetre above the brain slice and connected to a digitizer (USB TC08 Pico Technology). Room temperature was measured with the same type of probe but positioned outside the electrophysiology rig. The digitizer fed the information about the current temperature into a computer. The computer ran a Labview® based software (ETC, Patrick Spooner, Digitimer) that compared the temperature measurement with the target temperature set by the experimenter (32 degrees Celsius in our case) and sent an output signal to a dimmer unit (Soundlab DMX dimmer pack). This dimmer unit was modified to power a regular 1 KW heater placed underneath the electrophysiology rigs. In this way, the temperature was kept constant (± 0.1 degree Celsius). In the experiments described in this thesis, the incubation temperature was 32 ± 1 °C since colder temperatures are prone to epileptiform activity (Watson et al., 1997).

Temperature stability

By heating only the chamber and not the full electrophysiology rig, a temperature gradient between the surface of the electrodes and the moist oxygenated air produced condensation droplets (Figure 2.2A). When the droplets reached a critical volume, they fell onto the surface of the slice disrupting electrophysiological recordings (Figure 2.2B). Even thought the signal recovered after a certain delay the quality of the electrophysiology was strongly affected by these disturbances. All the stimulation pathways felt the effect of a water droplet falling on the slice and it took the fEPSPs at least 20 minutes to recover. This kind of disruption would be unacceptable during an experiment.

In order to avoid the problem of condensation droplets building up on the electrodes and moving them before finally falling on top of the slice, the whole

electrophysiology rig was heated. The goal was to prevent the condensation by reducing or eliminating the difference in temperatures between the warm and moist oxygenated air and the colder metal electrodes. Tests with regular heaters controlled by plug in thermostats provided the desired mean temperatures in the whole rig (Figure 2.3A). However, the output of the heaters had only two settings (on or off) and this produced cyclic ups and downs in the temperature that were then translated onto instability in our electrophysiological recordings (Figure 2.3B). During those small (± 0.5 degree C) oscillations, the size of the electrophysiological signal (slope of field EPSP) significantly correlated with the changes in temperature with a time delay of 5 min on average. The spatial resolution of these measurements was limited by the 2.5 min interval between test stimulation but the correlation informs us of the importance of the smooth control of the temperature.

With the implementation of our temperature control setup the temperature reached the experimental target temperature of 32 °C. The temperature was maintained constant irrespectively of changes in room temperature and within ± 0.1 degree for as long as the experiment lasted (Figure 2.4). Critically, the temperature is reached on the whole electrophysiology rig, eliminating the risk of condensation droplets. As expected, lifting the front screen of the electrophysiology rig to simulate access to the electrodes was detected as a sudden drop in temperature by the probes.

Constant humidity

In interface setups, the drying of the surface of the slice leads to an increase in the electrophysiological signal and this affects long experiments (Figure 2.5A). Reducing or increasing the airflow had a clear effect on the recordings (Figure 2.5B). These changes in excitability were slow (10-20% with respect to baseline every hour) but would compromise the interpretation of experiments lasting more than 3 h. To avoid these slow changes in humidity, at least three variables need to be controlled: the airflow rate, the level of distilled water in the under-chamber where the air is bubbled into, and the volume of air channelled onto the slice. The three variables are proportionally correlated with the humidity of the slice (i.e. higher air-flow, higher water volume and higher air volume produce an increase in slice

humidity). Manipulating these variables allows for the finding of the ideal settings for stable recordings and the monitoring and control of those values across experiments will facilitate their reproducibility.

The stabilization and control of both the temperature and the humidity inside the full electrophysiology rig allowed for over 16 hours of electrophysiological stability (Figure 2.6).

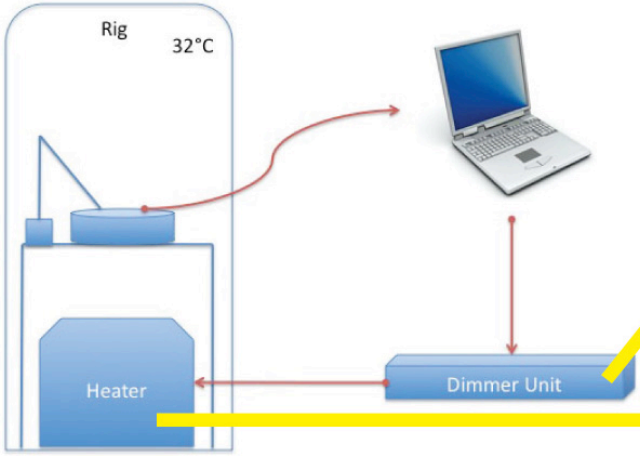
Fig 2.1 Rig, chamber and slice setup

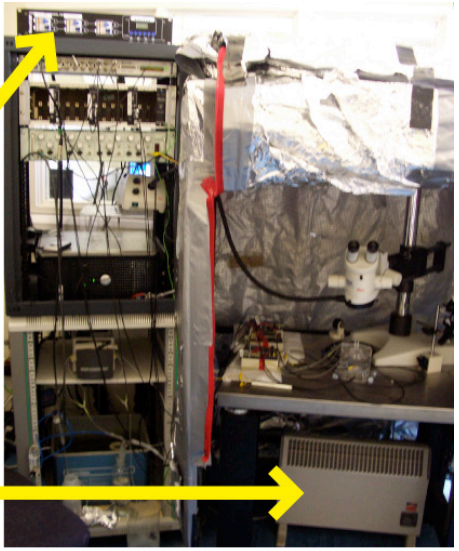
A, Schematic drawing of the new components responsible for the accurate control of the temperature of the electrophysiology rig (left panel) and picture of one of the rigs (right panel). The temperature probe is places as close to the hippocampal slices as possible and its reading is fed into a computer running the ETC software (Digitimer ©). The reading is compared to the desired temperature and through a modified dimmer unit, the right current intensity is sent to a 1 KW heater placed inside the rig, under the table. The aluminium foil wrapping of the Faraday cage insulates the inside of the rig.

B, Picture of the inside of the rig with a typical configuration of electrode manipulators (4) used to place 3 stimulating and 1 recording electrode around the CA1 area of the hippocampal slice (lower right insert).

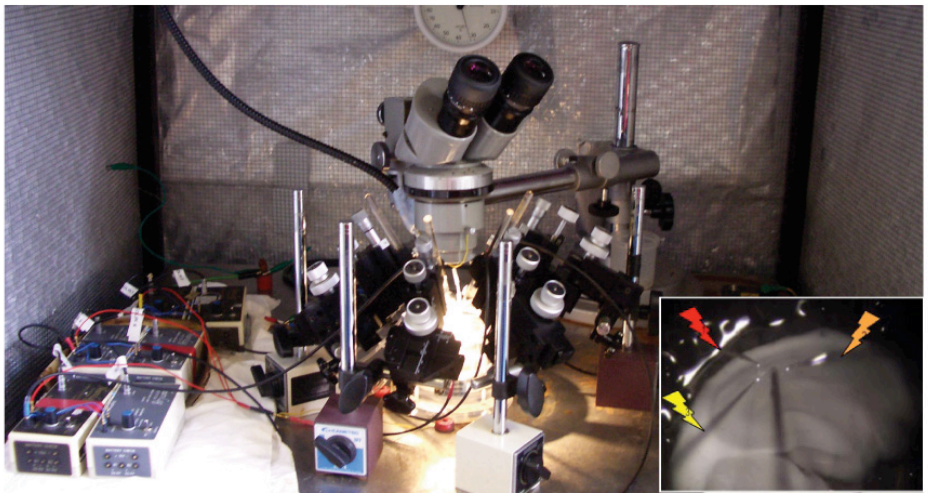
C, Schematic drawing depicting some of the modifications to the standard BSC2 interface chamber. Only one input hole is used for the inflow of aCSF. This hole is used to 'thread' the reference electrode through. At the other end of the chamber, another hole is used as the output of aCSF and the return electrode for the monopolar stimulation is threaded there. The flow of moist air (95% oxygen and 5 % carbon dioxide) is channelled through six holes and deflected onto the slices by a plastic cover (not drawn). The hippocampal slices are placed on top of 8 layers of lens tissue paper and up to 4 electrodes per slice are positioned as shown.

A


Rig
32°C
Heater
Dimmer Unit



B



C

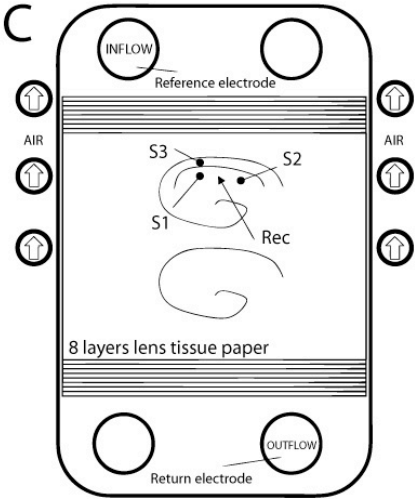

INFLOW
Reference electrode
AIR
AIR
S3
S2
S1
Rec
8 layers lens tissue paper
OUTFLOW
Return electrode

Fig 2.2 Effect of condensation droplets on electrophysiological recordings without whole-rig temperature control.

Temperature gradients and their effect on slice electrophysiology.

A) Moisture from the warmer oxygenated airflow builds up water droplets on top of the colder surfaces of electrodes and probes.

B) The periodic fall of these water droplets onto the surface of the slice immediately perturbs the recordings and creates an ionic imbalance of drastic biochemical consequences.

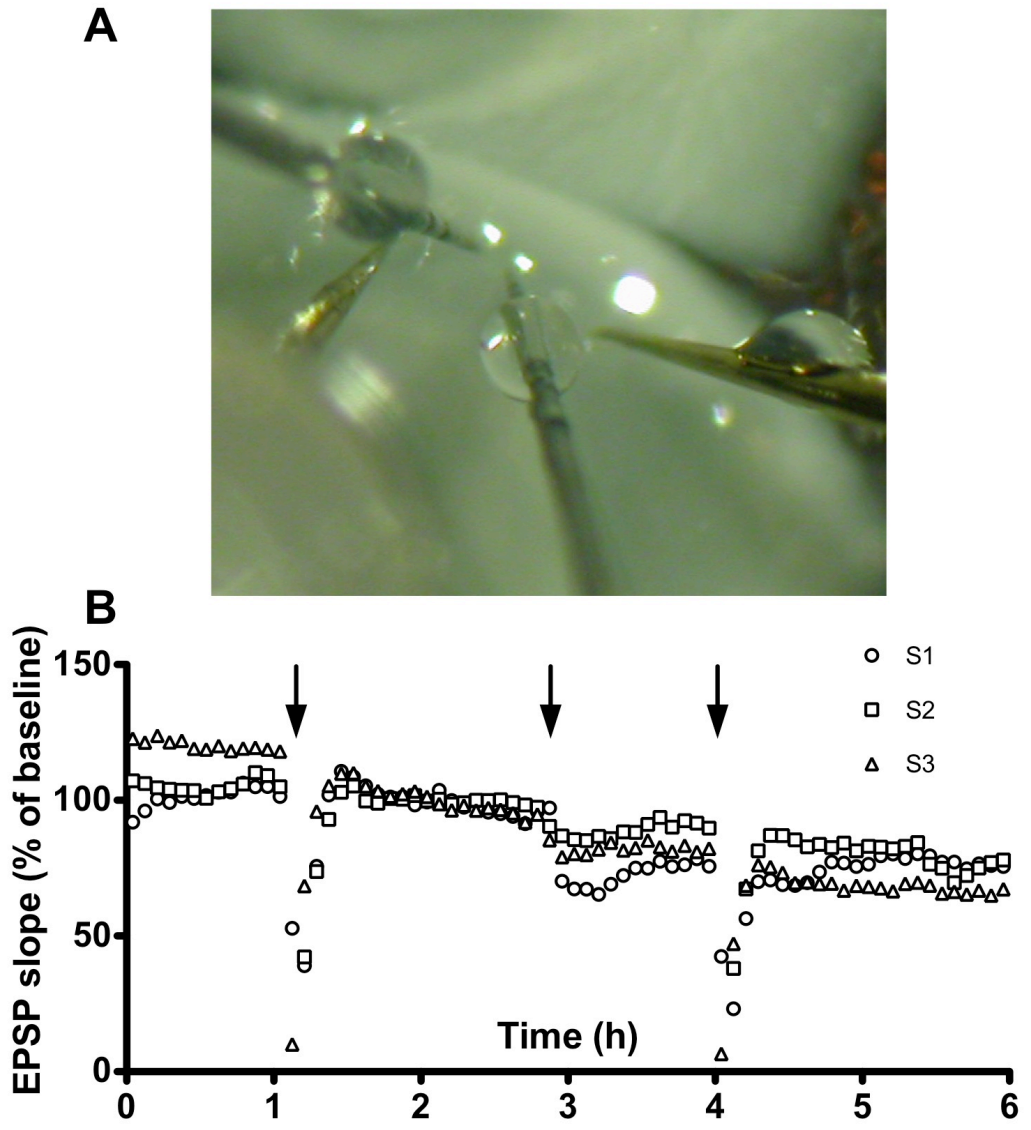


Fig 2.3 Effect of temperature changes on electrophysiology when heating the whole rig without the ETC software.

A) Plug-in thermostats were initially used to control the output of the heater and this panel shows the temperature oscillations during one hour of experiment. The use of 1 KW heaters to warm the full rig without good control on their input produces oscillations in the temperature.

B) The oscillations in temperature shown in (A) correlate with oscillations in the fEPSPs recorded in area CA1. Cross-correlation between the changes in temperature and the size of the EPSP slope (measured every 2.5 min). The slope of the EPSP correlates maximally with the temperature after a delay of 5 min.

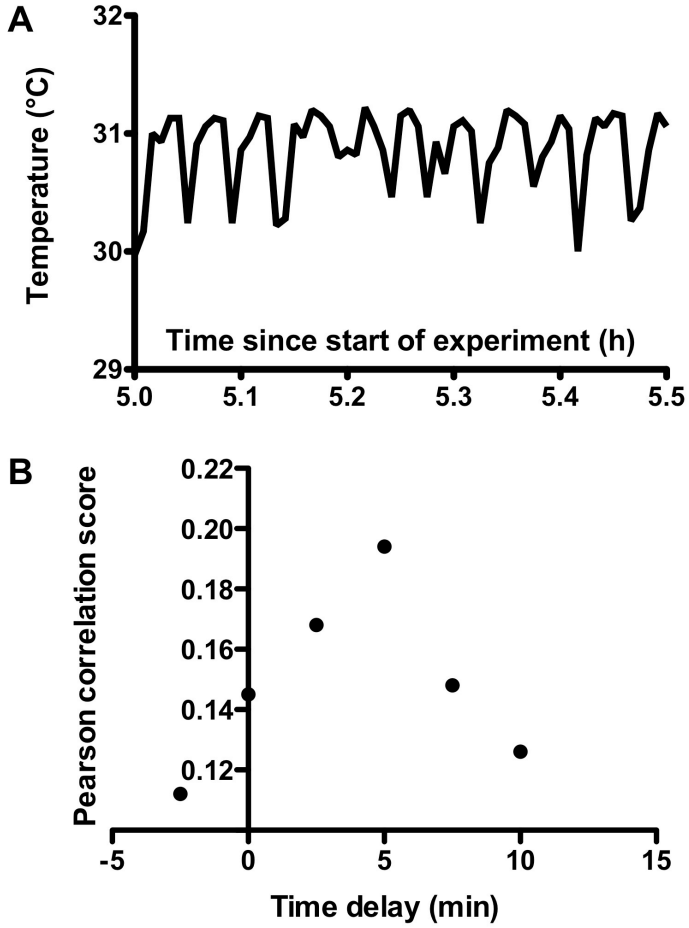


Fig 2.4 Temperature inside the electrophysiology rig under ETC control is independent from room temperature.

A) With the ETC temperature control developed as part of this thesis, the temperature inside the rig (upper panel) remains unaffected by variations in room temp (lower panel).

B) Expanded 2 hour period showing the degree of accuracy in the control of the temperature (± 0.1 degree max.)

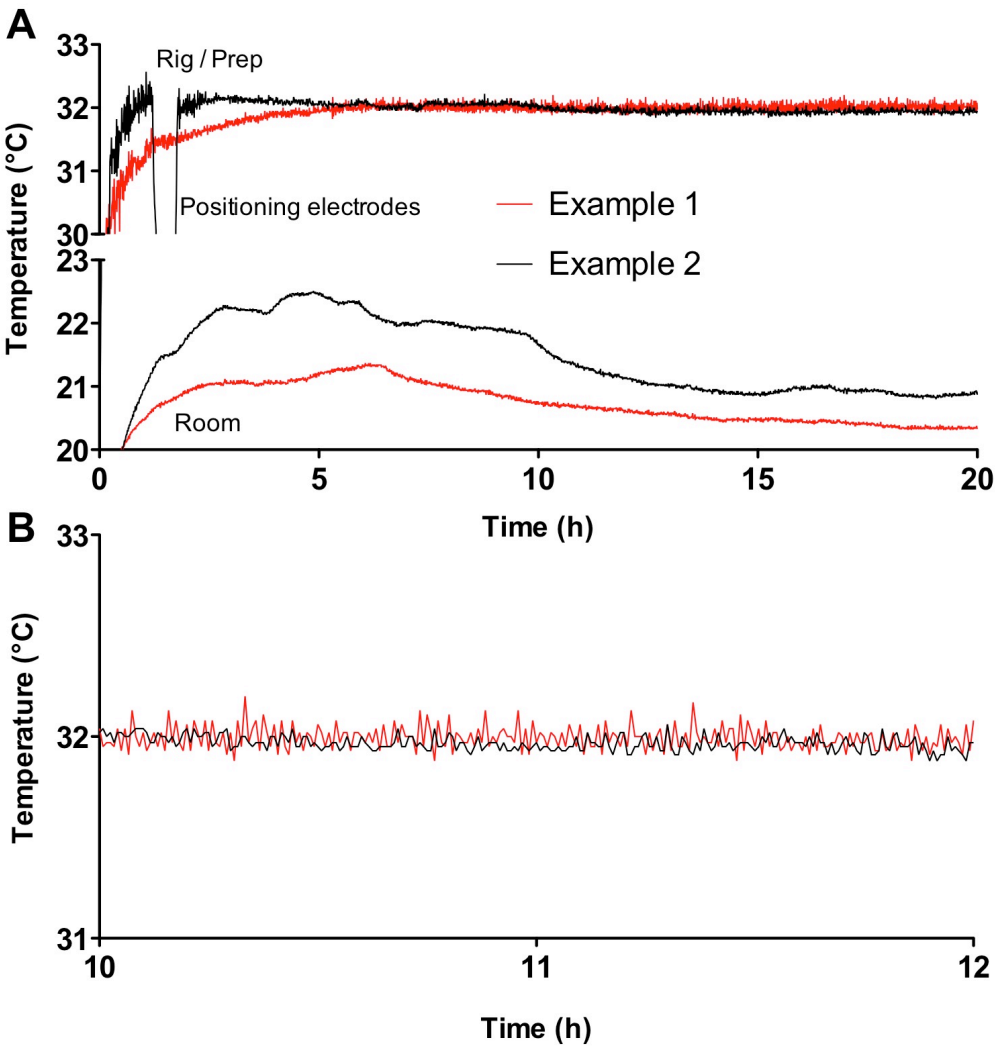


Fig 2.5 Effects of humidity changes.

Changes in surface humidity lead to changes in electrophysiological measurements.

A) As the surface of the slice dries out, its electrical resistance will increase so as to extend the reach of stimulation of constant intensity. This will be reflected in an ever-increasing baseline fEPSP recording as more fibres are recruited by the stimulation.

B) Changes in the flow of moist air on top of the slice alter that resistance and have a clear effect on the recording of fEPSPs.

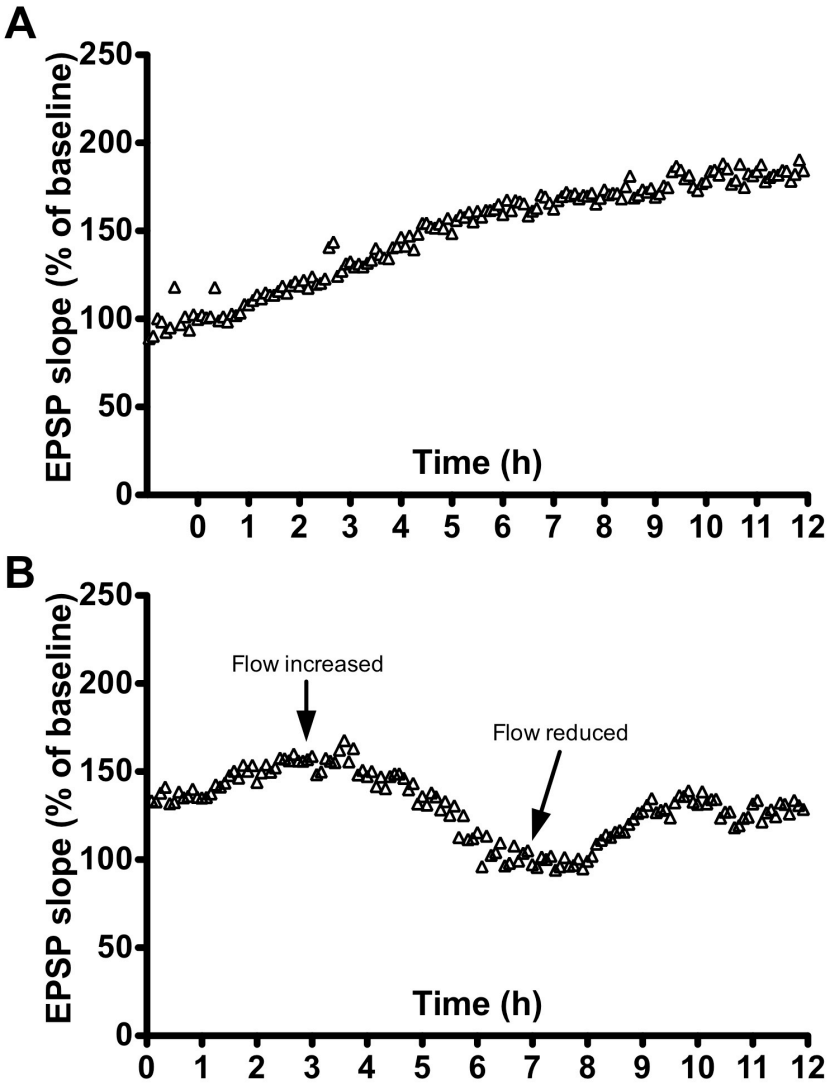
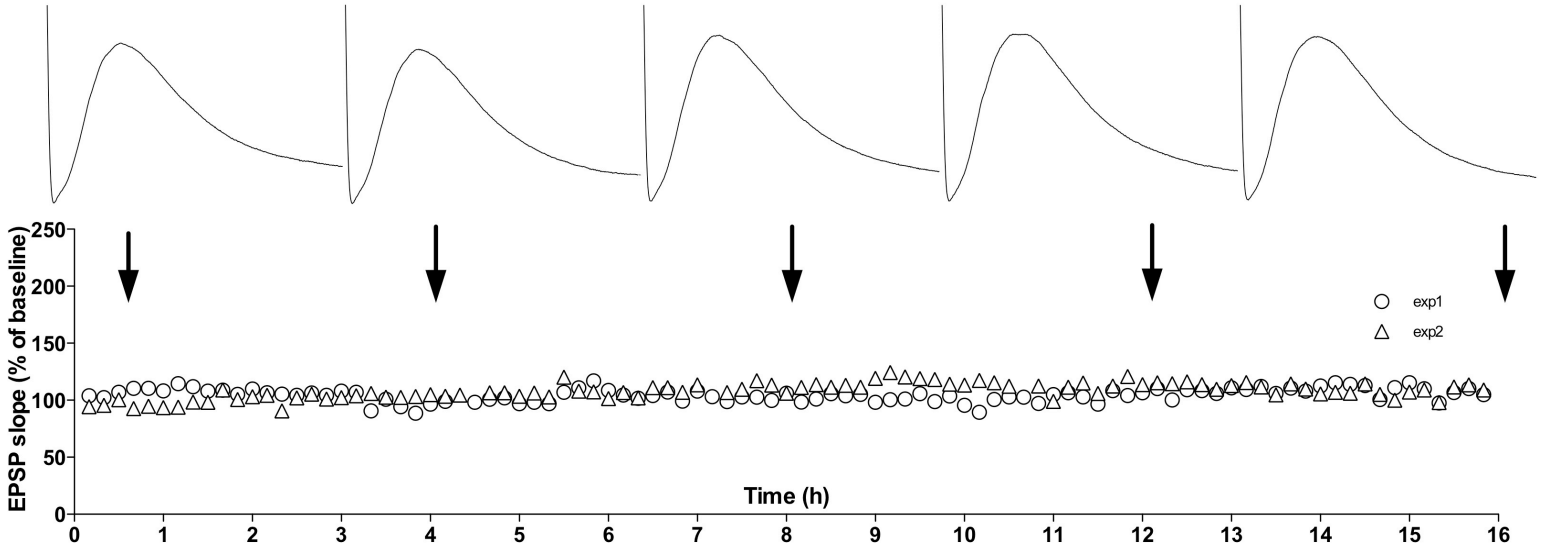


Fig 2.6 Stable baseline.

End result from the combination of constant temperature control on the whole electrophysiology rig and careful stabilization of the humidity levels on top of the brain slice.

A) **Traces from a control pathway and some representative baselines can be obtained routinely after implementation of the aforementioned techniques.**

B) **The stability of the slice allows for the study of the very late phases of physiological phenomena (LTP).**



2.2 The Event arena

The behavioural experiments described in chapters 8 and 9 involve the use of a dry maze to test the spatial memory of rats in an attempt to connect the STC hypothesis to behaviour. There are some differences in the methodology used in every experiment and those differences are described within the methods sections in their respective chapters. What follows is a description of the common procedures dealing with the event arena.

Male Hooded Lister rats (250-430g) were housed in stainless steel cages. The rats were kept at 90 % of free-feeding body weight and food deprived for 18-22 hours before each training or test trial. All rats were maintained on a 12 h light/12 h dark schedule, with testing occurring in the light phase.

All experiments were conducted in a square Perspex event arena (165x165cm) lined with sawdust and containing two colourful intramaze cues (a 'pyramid' and a 'spaceship', both approximately 40cm high). Four computer-controlled entrances, one along each wall, were connected to Perspex start boxes covered on the sides, but not the top, with black card for animal comfort. Sand-filled Perspex wells (diameter 6cm, depth 3cm) could be inserted into the floor of the arena through uncovering of sawdust and lifting of small plastic lids at a total of 49 possible locations; where no wells were inserted the lids were hidden from view by the sawdust (not pictured). The arena was situated at table height in a rectangular room. Prominent extramaze cues were mounted on the surrounding walls. A screen on one side of the room allowed animals to be carried into the experimental room and placed on an adjacent trolley without seeing the event arena. Two video cameras, mounted on the ceiling and the wall, allowed for tracking and close-up views of the animal from outside of the experimental room. Lighting was kept constant at 115 lux to ensure sufficient lighting but minimal stress to animals.

Behavioural Protocol for the Delayed Match to Place task in the event arena.

Shaping and Habituation

During the first week, the food-deprived animals were taught to dig for food in wells by receiving some of their ration in sand wells in their cages. Over the next six days, they were habituated to digging in the event arena. On the first day, they were placed into the centre of the arena and were allowed to explore for 5 min; on successive days, they were placed in start boxes, and after a 20 second delay were allowed into the arena where a half-pellet food reward was buried in a sandwell. Half-pellet rewards were initially buried close to the surface and then progressively deeper.

Pre-Training

In this phase, animals were taught the one-trial place memory task in the event arena previously described by Bast et al. (Bast et al., 2005) with some modifications. Trials consisted of an encoding phase (SAMPLE) and a retrieval phase (CHOICE). The sample phase was conducted exactly as during habituation, with the rat spending 20 seconds in the startbox before being let out into the event arena where a single, rewarded well was uncovered. The rat was allowed to dig for the reward and then eat the half-pellet in its home cage. After the sample phase, a delay that depended on the experiment (see individual methods sections) was introduced.

In the choice phase, the same well again contained food, but was now presented along with four unrewarded wells so that the animal had to use one-trial place memory according to a win-stay rule to most efficiently retrieve the half-pellet. The location of the rewarded well was changed daily in a pseudo-random fashion. In order to prevent the use of smell to find food, sand in every single well contained 5% of pulverised pellets. Only one sample-choice trial was given on each day for each animal. Performance was measured in terms of the number of errors made before reaching the rewarded well. Latency to dig in the well in both the sample and choice phases was also recorded. When performance was consistently above chance (average of less than 2 errors across all animals, whereby an error is defined by digging in an incorrect well prior to the correct well), the experiments started.

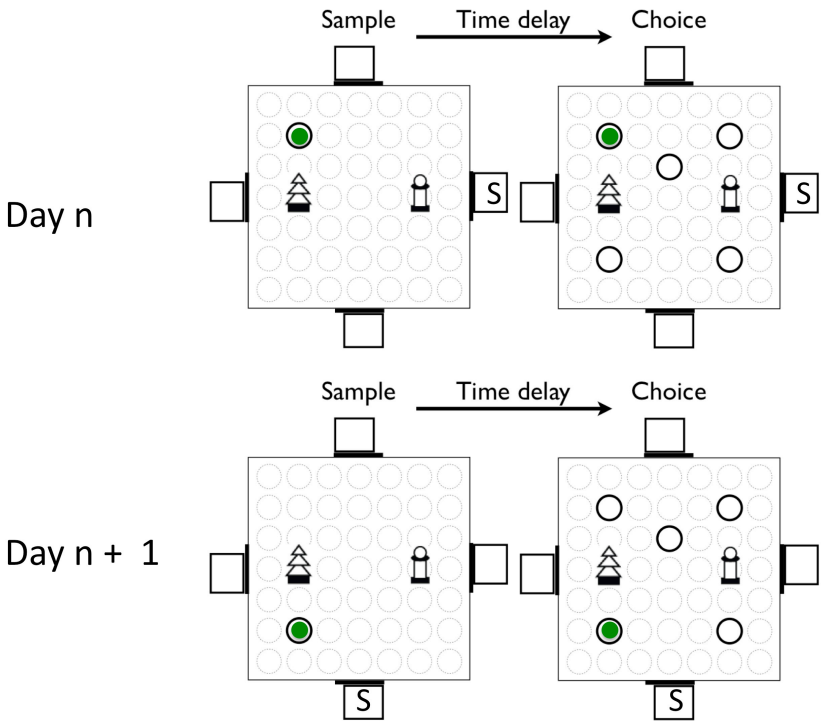
Training days were further added in between test days in order to maintain performance.

Figure 2.7: The Event Arena Training Protocol.

Training trials consisted of an encoding phase (SAMPLE) and a retrieval phase (CHOICE). In the sample phase the rat spent 20 seconds in the startbox before being let out into the event arena where a single, rewarded well was uncovered. The rat was allowed to dig for the reward and then eat the half-pellet in its home cage. After the sample phase, a delay that depended on the experiment (see individual methods sections) was introduced.

In the choice phase, the same well again contained food, but was now presented along with four unrewarded wells so that the animal had to use one-trial place memory according to a win-stay rule to most efficiently retrieve the half-pellet. The location of the rewarded well was changed daily in a pseudo-random fashion. Only one sample-choice trial was given on each day for each animal. Performance was measured in terms of the number of errors made before reaching the rewarded well. Latency to dig in the well in both the sample and choice phases was also recorded.

Green dot: location of reward; S: Start box for the particular day.



Novelty exploration

Over the course of two experiments, described below, four different novelty 'treatments' were used, designed to induce protein synthesis in the hippocampal cells (Vazdarjanova *et al.,* 2004). In each case, treatment consisted of a five minute exploration phase in either one of two differently-shaped Perspex boxes placed within the event arena (intramaze cues were moved to the side of the arena in all cases). When a box was experienced by the animals more than once, we kept the novelty of the experience by introducing different substrates on the floor. This included mesh wire, bubble wrapping paper, cotton pads, shredded paper, shredded straws, clear pebbles (~2cm diameter, 1cm thick), plastic dinning mats, anti-slip mats, and coloured stones (<1cm diameter, 0.5cm thick). Boxes and room are depicted in Figure 2.8, below.

Figure 2.8: Box exploration: The triangular box.

The box was placed within the event arena. The walls were made of Perspex to allow the animal to look out at the event arena.

2.3 The Morris Watermaze

The behavioural experiments described in chapter 10 use the Morris watermaze (Morris, 1984) to test the spatial navigation memory and its modulation. This task is hippocampal dependent (Morris et al., 1982) and has been used extensively to test spatial memory. There are different versions or tasks that can be run in the watermaze and the experiments in chapter 8 rely on the delayed-match to place task described below.

The watermaze is a fibreglass pool of 2 m in diameter and 60 cm in height, whose inside walls are coated with gelcoat and painted white. It sits in the middle of a room with prominent extra-maze cues. The water reaches a level of approximately 40 cm. The swimming rat can escape onto a solid platform, 11 cm in diameter, whose surface is only 1-2 cm below the water's surface. The submerged platform is invisible for a swimming rat as the water is made opaque by adding white latex. The rat's path, recorded by video camera, is fed to an image analyser with the coordinates sampled at 10 Hz by a computer running Labview Software. The program analyzes different measures such as the escape latency (i.e. the time taken by the rat to find the platform), the path length, the percentage of time spent near the side-walls, the swim speed, or the percentage time spend in a specified area of the pool.

During testing, the water is maintained at 25 ±1 °C. In a normal trial, the rat is placed in the water facing the walls at any four prearranged positions corresponding to the four cardinal points (N-S-W-E). The rat then swims in search of the platform. The rat is allowed to sit on the platform for 30 s to allow it to look around and associate the platform position with the extra maze cues. At the end of each trial the rat is dried with a towel and placed in a cage.

The delayed-match-to-place task

In this task the rat receives four trials per day to learn a new platform position each day (Steele and Morris, 1999). The platform position chances pseudo-randomly from day to day. This means that on the first trial of any day the rat has no information about the current platform location. On subsequent trials (trials 2 to 4) the location of the platform remains constant and the rat learns the win-stay rule. Once the rule has

been learned, the performance on trial 2 is a measure of the rat's one-trial memory while trials 3 and 4 remain as tests of rule learning as well as reinforcing the strategy.

At least three measures can be analyzed in this task. We used the Atlantis platform which consists of a regular platform that is held deeply submerged, out of reach of the swimming rat, by magnets. After the rat has spent a determined amount of time (i.e. 60 s) swimming, the platform is raised and the rat allowed to mount on it. During a probe trial, the tracking of the rat as it swims during those 60 s allows for the quantification of the time spent inside a certain area around the platform (30 cm diameter circle). This time can be given as percentage of the total time (60s) and is one of the measures most often used in the DMP task. The percentage time around the correct location improves over the measure of latency to reach the platform location. It does so because the latency can be artificially short by a false positives when rats bump on the platform by chance while swimming. On the other hand, the Atlantis platform and the percentage measure that it allows, avoid this source of variability. The data in chapter 10 will be presented both as percentage of time around the correct location and the latency to reach it, which measures the latency to swim over the location of the now submerged Atlantis platform.

A third measure of performance in the DMP task relies on the amount of time that a particular rats shortens its latency to swim to the correct location from the first trial (when the rat ignores to new location for the day) to the second trial (when memory performance is measured). The 'savings' measure is calculated by substracting the latency of trial 2 from the latency in trial 1.

2.4 Surgical procedures

The delivery of drugs used in chapter 9 involved the bilateral implantation of cannulae into the dorsal hippocampus. Rats were anaesthetised with isofluorane. The animal was then placed in a stereotaxic frame with the mouth bar placed such that bregma and lambda co-ordinates would rest in the horizontal plane. An incision was made longitudinally in a line mid-way between the ears. The skin was held at the sides with forceps exposing the top of the skull. The co-ordinates for implantation were calculated from bregma and marked over the skull. A dental drill was used to remove the bone over the implantation points. The animals were implanted bilaterally with permanent 26 gauge steel guide cannulae. The coordinates were based on the stereotaxic plates of Paxinos and Watson atlas; anterior-posterior coordinate -4.5 mm refers to bregma, lateral coordinate +- 3.0 mm to the midsagistal suture line and ventral coordinate -3.9 mm (injection site) to the surface of the skull. The guide cannulae were secured to the skull with dental cement and miniature screws. The skin was sutured around the head cap. A dummy cannula was kept into the guide cannula and the rats were given at least 5 days of recovery in the home cage.

Microinfusions

An infusion pump was used for bilateral infusion at a flow rate of 0.25 μl/min. Microsyringes (5 μl, SGE, Australia) were mounted on the pump and connected with injection cannulae (33 gauge, 0.5mm beyond the guide) using flexible polyethylene tubes. During infusions, the rats were restrained lightly with a towel. The injection cannulae were left in the guide cannulae for 1 min after the infusion. Once the injection ended, the dummy cannulae were placed back into the guide cannulae.

For behavioural studies, SCH23390 hydrochloride (Tocris, UK) was dissolved in sterile normal physiological saline and kept in frozen aliquots (500 μl) until usage. SCH23390 final concentrations of 1 mg/ml or 3.3 mg/ml were used in this study. Anisomycin (Sigma-Aldrich, UK) was dissolved in 1N HCl, diluted with sterile normal physiological saline and adjusted to pH 7.4 with 1N NaOH to produce a final concentration at 125 mg/ml. d-AP5 (Sigma-Aldrich, UK) was dissolved in normal

physiological saline and adjusted to pH 7.4 to produce a final concentration at 5.9 mg/ml.

2.5 Statistical analysis

Electrophysiology

The average values of the slope function of the field EPSP (fEPSP) (mV per ms) for each time point were analyzed using paired (within-group) and unpaired (between group) t-tests; $p < 0.05$ was considered as statistically significant, but we show more exacting levels of significance in many cases. Parametric tests were used as the data conformed to a Gaussian distribution, but analysis using non-parametric tests (Mann-Whitney and Wilcoxon tests) gave the same results. In order to measure the stability of L-LTP, we compared the levels of potentiation 2 h after its induction with those levels remaining after 10 h. This measurement distinguishes between stable L-LTP and decaying forms of LTP that still show potentiation at the end of the experiment because of a strong initial expression. We hope that this measurement clarifies the difference between strength and persistence of LTP.

Behaviour

When appropriate, ANOVA was used to confirm differences between conditions. In most cases, the experiments were run in a within-subjects design that allowed for the benefit of repeated measures and paired tests. In addition to the ANOVA test, multiple comparisons under Bonferroni corrections allowed the investigation of specific statistical differences between conditions and treatments.

In the event arena tasks, the percentage of time spent digging in the correct well could be compared to chance performance (defined as 20% of the dig time spent in the correct well). Performance above the 20% chance level denoted some memory for the correct location. This allowed conditions to be compared against the chance level for no memory, in addition to being compared one to each other.

Chapter 3: Induction of late-phase LTP (L-LTP)

3.1 Introduction.

Late LTP is defined as a long lasting potentiation that requires *de novo* protein synthesis and that lasts more than 3 h (Kelleher et al.). This time point is not standard. Originally in the literature, and particularly for intracellular recordings, LTP that is sustained for more than 1 h is reported as late LTP. On the other hand, with the perfection of long lasting recording techniques the 3 h band has been extended to 4, 6, or even 8 hours.

The mode of induction of LTP can determine both its initial strength and persistence. The simple model of Huang et al. (1994) whereby the distinction between E-LTP and L-LTP was limited to whether a single train or multiple trains were delivered is outdated (Huang and Kandel, 1994). Sajikumar et al 2008 and my personal observations as shown below demonstrate that a single tetanus may be enough to induce L-LTP (Sajikumar et al., 2008).

There are two main methods of inducing LTP by electrical stimulation. Some groups prefer to employ the classical 100 Hz stimulation protocol while varying the number of trains and inter-train interval (from 30 s to 10 min). The alternative to 100 Hz stimulation is the use of short bursts of 4 to 5 pulses delivered at 100Hz and repeated at 5 Hz in concordance with the theta frequency detected in EEG and associated with exploratory behaviour. These short bursts at 100 Hz mimic the firing pattern of CA3 pyramidal cells (Brown and Randall, 2009) and the 5 Hz frequency of the burst may benefit from the shutdown of feedback inhibitory inputs. The first aim of my research was to develop a reliable induction protocol capable of producing NMDAR and protein synthesis dependent L-LTP.

3.1.1 Aim of experiments.

Experiments in this chapter are aimed to obtain a reliable induction protocol for a NMDAR and protein synthesis dependent L-LTP in the Schaffer collateral projections from CA3 to CA1. Because the overall thesis aims to contribute to the

understanding of synaptic tagging and capture, the initial and main L-LTP induction protocol used was three trains of 100 pulses at 100Hz delivered at 10 min intervals, which is the protocol used in earlier STC experiments.

In the experiments described in this chapter, the protein synthesis dependency of L-LTP was tested with the use of the protein synthesis inhibitor Anisomycin and the NMDAR dependency was tested with the NMDAR antagonist D-AP5.

3.2 Methods

3.2.1 Preparation of slices and recording set-up

Experiments were conducted with hippocampal slices prepared from 6 to 8 week old male Wistar rats. Slices were prepared as described in chapter 2 and maintained at 32 °C in an interface recording chamber. Two stimulating electrodes were positioned in the *stratum radiatum* of CA1, each 500 μm away from the recording electrode placed in between (Figure 3.1). An additional stimulating electrodes was placed in the *stratum oriens*. With this configuration it was possible to evoke responses from three independent but convergent input pathways (S1, S2 and S3 respectively) and record the extracellular field EPSP response to stimulation of each pathway. The independence of each pathway was established by lack of paired pulse facilitation between the pathways (data not shown) and also by determining whether there was any post-tetanic potentiation (PTP) in the non-tetanised control pathway following high frequency stimulation of the tetanus pathway.

3.2.2 Tetanus protocols

For the standard induction of L-LTP, a tetanic stimulation protocol consisted of high-frequency tetanic trains at 100 Hz in 1 s bursts, given three times with an inter-train interval of 10 min (biphasic pulses with 100 μs per half-wave) was used. This 3x (100p 100Hz) 10 min ITI is defined as the 'strong tetanus' in this thesis.

As stated earlier, the criterion for exclusion of experiments was a drift of > 30% either upwards or downward within the period of the experiment. Recordings were made for a minimum of 10 h post-tetanus, thus a drift of 30% corresponds to a mean change of 3% per hour.

3.2.3 Drug preparation

Anisomycin

Anisomycin was dissolved in equimolar HCl, diluted with aCSF and adjusted to pH 7 with NaOH to produce a final concentration of anisomycin of 125 mg per ml. For slice electrophysiology, a stock solution of anisomycin was prepared as described in chapter 2, frozen and diluted in aCSF to the final concentration of 25 μM before allowed to circulate through the slice electrophysiological preparation.

D-AP5

The NMDA receptor antagonist (2R)-amino-5-phosphonovaleric acid (D-AP5) was prepared as a stock solution at 1 mM. The drug was dissolved in aCSF on each day of an experiment to a final concentration of 25 μM.

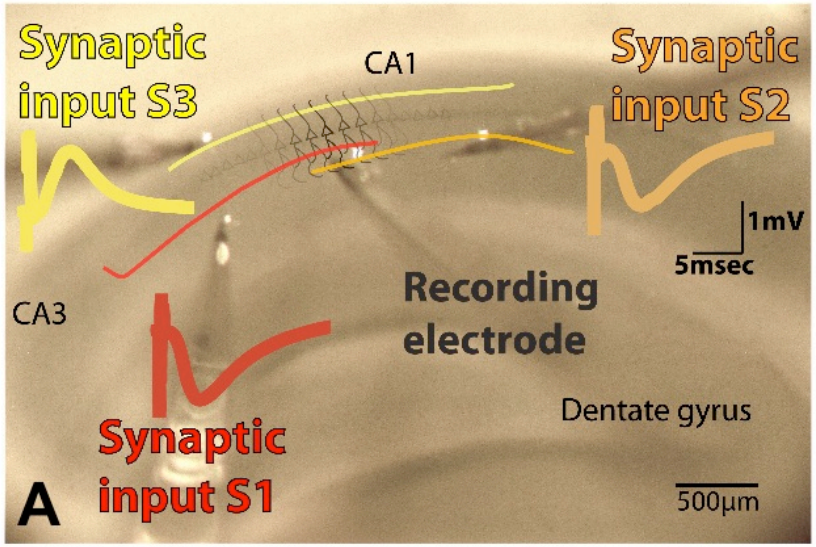
Figure 3.1 Picture and schematic representation of the *in vitro* hippocampal slice preparation.

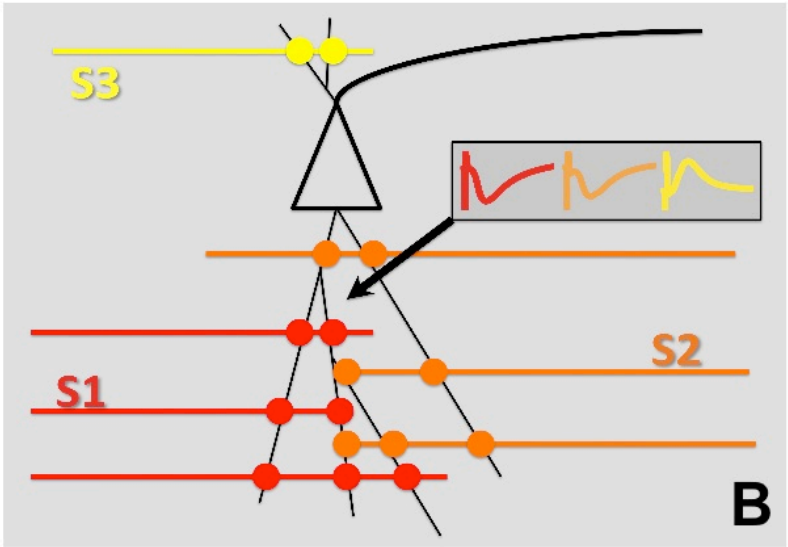
A, The slice preparation with superimposed labels depicting the positioning of the electrodes. Colour-coding matches that used to identify the respective pathways throughout this thesis.

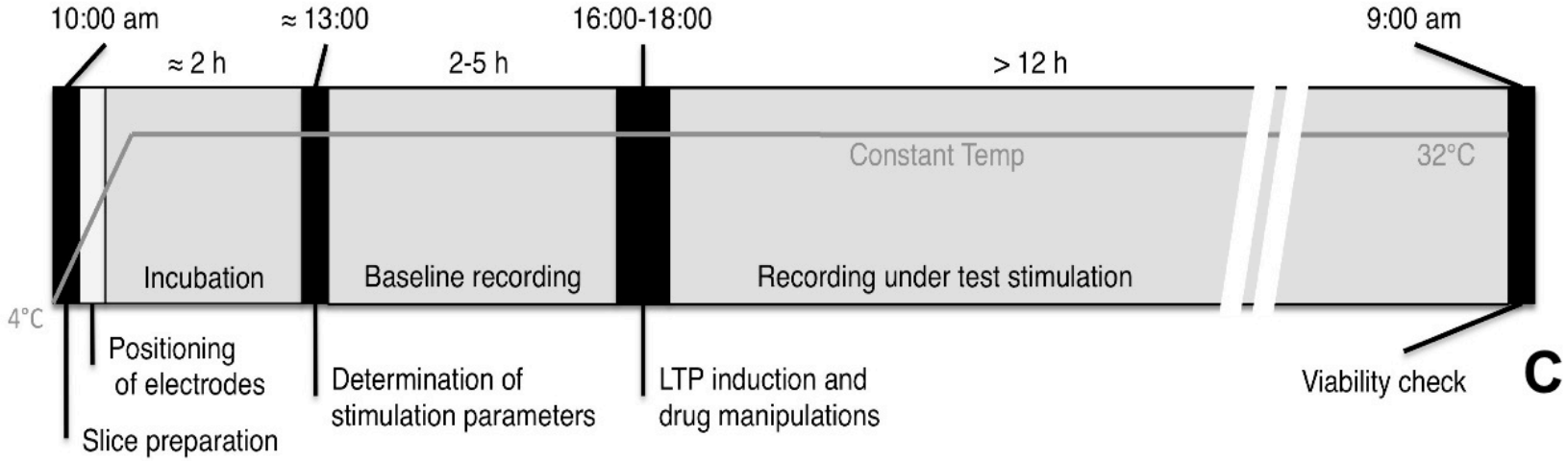
B, Schematic representation depicting the independent but convergent inputs onto pyramidal cells in the CA1 used in these experiments. The recording electrode placed in the *stratum radiatum* of CA1 records three independent field EPSPs elicited by the activation of different populations of synapses onto the same cells.

C, Experimental protocol showing approximate phase lengths. Briefly, slices are cut in ice-cold aCSF and transferred to the recording chamber where the electrodes are placed in position. 2 hours of incubation allow the temperature to equalize to 32 °C before input/output curves and paired pulse stimulation tests are run to assess the optimal intensity of stimulation and confirm pathway independence, respectively. More than 2 hours are still allowed to pass while baseline recordings are obtained before any drug or electrophysiological manipulations are introduced. After that, the setup returns to test-stimulation frequencies and the experiment is allowed to develop overnight. An assessment of the viability of the slice is run the following morning (see methods section for more detailed information).

Abbreviations: CA1 = Cornus Ammonis area 1. CA3 = Cornus Ammonis area 3.


Synaptic input S3
CA1
Synaptic input S2
1mV
5msec
CA3
Recording electrode
Dentate gyrus
Synaptic input S1
500µm
A


S3
S2
S1
B


10:00 am
≈ 13:00
16:00-18:00
9:00 am
≈ 2 h
2-5 h
> 12 h
Constant Temp
32°C
Incubation
Baseline recording
Recording under test stimulation
4°C
Positioning of electrodes
Slice preparation
Determination of stimulation parameters
LTP induction and drug manipulations
Viability check
C

3.3 Results.

3.3.1 Assessment of maximal response (input/ output measurement).

After the positioning of the electrodes and the required waiting period for the stabilization of the slice, the test stimulation intensity for each pathway was determined by conducting and input/output curve. Test stimuli (biphasic square-wave pulses 0.1 μs per half-wave were delivered to each pathway once every 30 s), were applied to all S1, S2 and S3 pathways at a range of stimulus intensities (from 50 to 300 μA in 50 μA steps). Intensities capable of eliciting 40 to 50 % of the maximum response were used as the test stimulus intensities during the remaining of the experiment. Across all experiments included in this thesis, the average response over this range of stimulation intensities showed slope functions of -0.25 +/- 0.09 mV/msec across all 157 pathways monitored in *stratum radiatum* and +0.20 +/- 0.01 mV/msec across the 92 pathways monitored in *stratum oriens*.

In all experiments, stable baseline responses were recorded for at least 2 h prior to high-frequency tetanisation. The overall excitability of the preparation was monitored through the use of a non-tetanized pathway.

3.3.2 The induction of L-LTP in hippocampal CA1 area.

Representative field EPSPs of the pathway of interest are shown from an individual experiment in Fig 3.2. Control traces are identical to those depicted in the Chapter 2 Fig. 2.6.

An individual example of one of the experiments is shown in Figure 3.3A and the data is grouped in Figures 3.3B & C (n = 7). The tetanus (i.e. 3 x 100p at 100Hz, 10 min inter-tetanus interval) was delivered to the S1 pathway and 10 min post-tetanus, the average level of potentiation was 212%. This response declined to 166% of the pre-tetanisation level after 2 h but then stabilized at 147% after 6 h and 141% at the end of the experiments 10 h post-tetanisation. This stabilization is represented graphically in Figure 3.3C.

In summary, the tetanised pathway (S1) showed robust potentiation after the three high-frequency tetanic trains. LTP lasting > 10h with an absolute level of around 44% relative to both the pre-tetanisation baseline ($t = 8$; $p < 0.01$) and a non-tetanised control pathway ($t = 4.9$; $p < 0.01$) was routinely obtained by application of the 'strong tetanus' (Fig. 3.3B). This L-LTP was stable over time (comparison of 2 h and 10 h time-points; $t = 2$; $p > 0.05$).

Figure 3.2 Representative example of fEPSP waveforms from an individual experiment with high frequency stimulation to induce L-LTP.

Individual fEPSPs from the stimulated (S1) pathway recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 3.3A

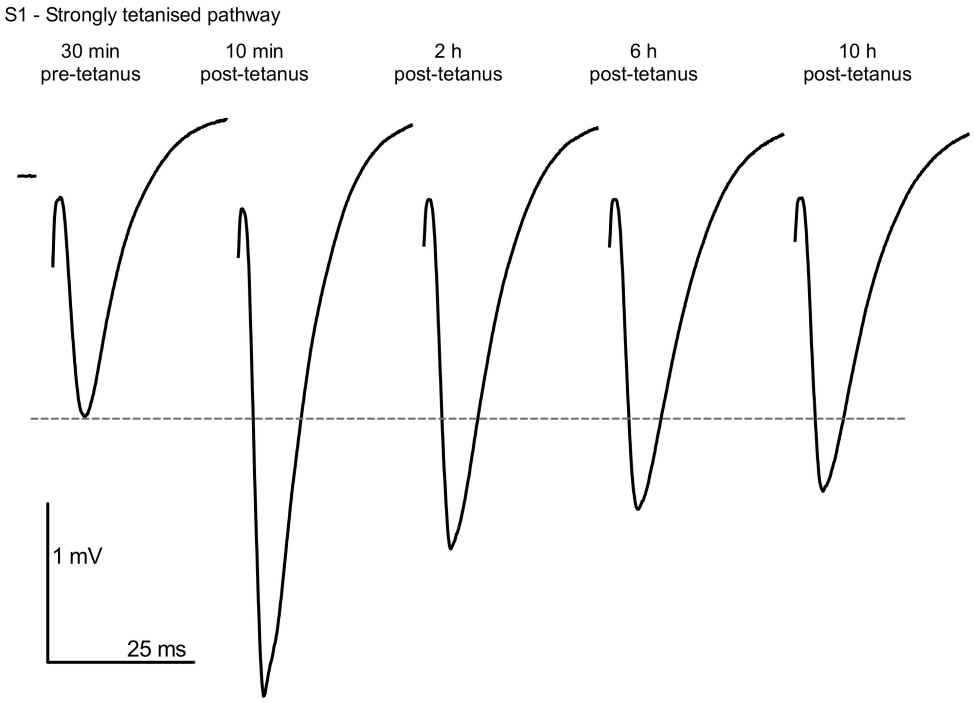
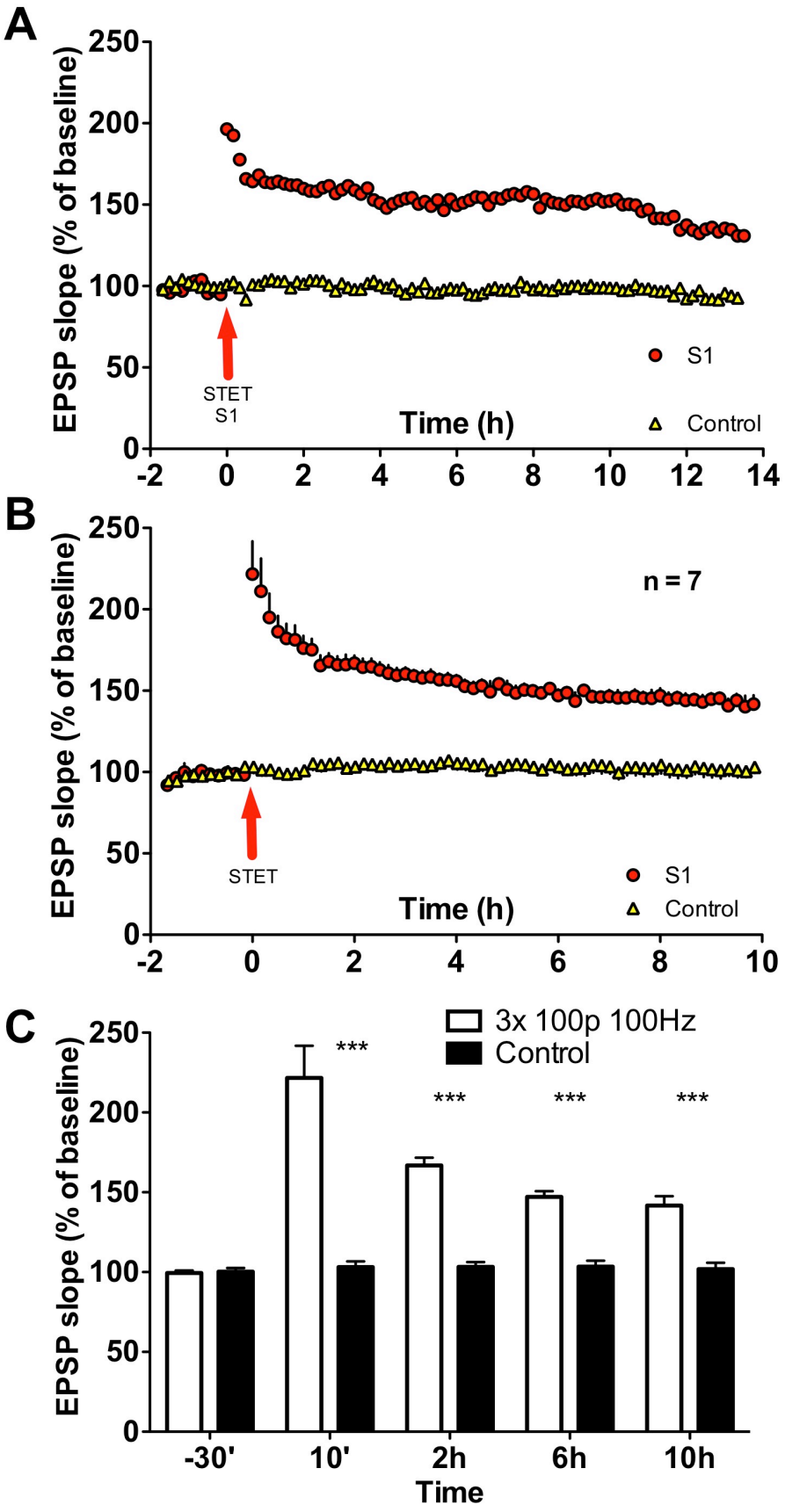


Figure 3.3 Induction of L-LTP in CA1 of the hippocampal slice.

A. An individual experiment showing a representative example of the induction of L-LTP.

B. Grouped data for experiments in which L-LTP was induced (n = 7). Field EPSP slope is expressed as the percentage change normalised to the pretetanus baseline level. Baseline (100%) was taken as the mean of the values obtained for 60 min of pre-tetanus test stimulation. Error bars indicate ± s.e.m. Symbols: S1 tetanised pathway ●. S2 control pathway △

C. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired student t – test, with Bonferroni correction for multiple comparisons). Significant differences were observed at all times.**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
12
14
STET
S1
Time (h)
S1
Control
B
EPSP slope (% of baseline)
n = 7
STET
Time (h)
S1
Control
C
EPSP slope (% of baseline)
3x 100p 100Hz
Control
***
***
***
***
-30'
10'
2h
6h
10h
Time

3.3.3 L-LTP is sensitive to 25 μM Anisomycin.

L-LTP is defined as a protein-synthesis dependent stage in the maintenance of potentiated synaptic changes. This protein synthesis dependency is an intrinsic part of the STC hypothesis that this thesis deals with. Therefore, the tetanisation used in this chapter needs to elicit protein-synthesis dependent L-LTP. To assess this, the protein synthesis inhibitor anisomycin was allowed to act on cells during the induction of L-LTP. 25 μM anisomycin present around the time of induction did not have an effect on the early stages of LTP but blocked the maintenance of L-LTP in a way similar to that described previously (Krug et al., 1984). L-LTP declined to baseline after 10 hours since there was no difference between S1 before the tetanus and S1 10 h post-tetanus ($t = 0.8$, $p > 0.05$) nor was a difference between S1 and the control pathway 10 h post-tetanus (S1 vs. baseline; $t = 0.14$, $p > 0.05$). There was, furthermore, a decline in the level of potentiation in S1 from 2 h and 10 h ($t = 4.956$, $p < 0.01$).

Figure 3.4 Representative example of fEPSP waveforms from an individual experiment with high frequency stimulation under the presence of 25 μM Anisomycin.

Individual fEPSPs from the tetanus (S1) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 3.5

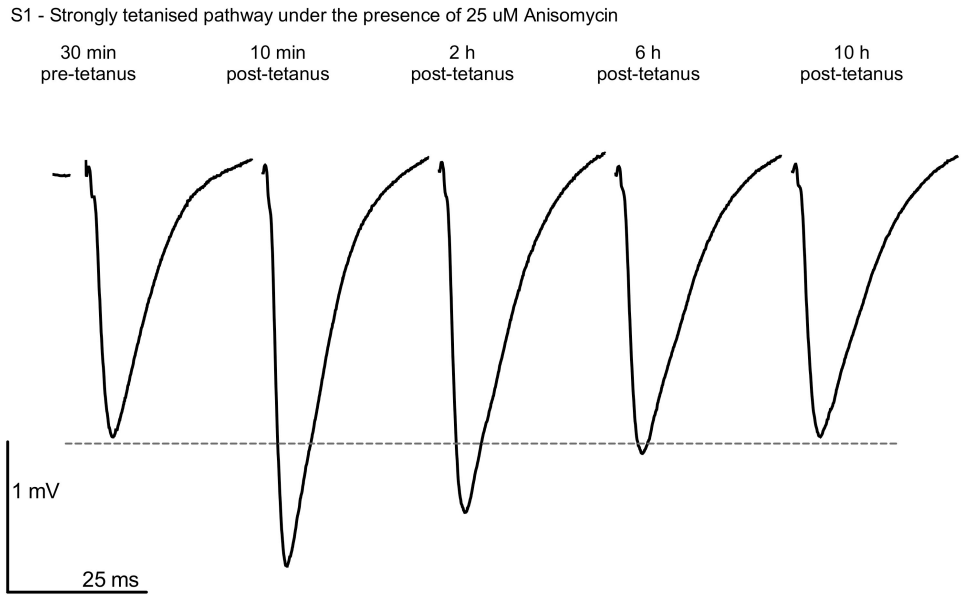
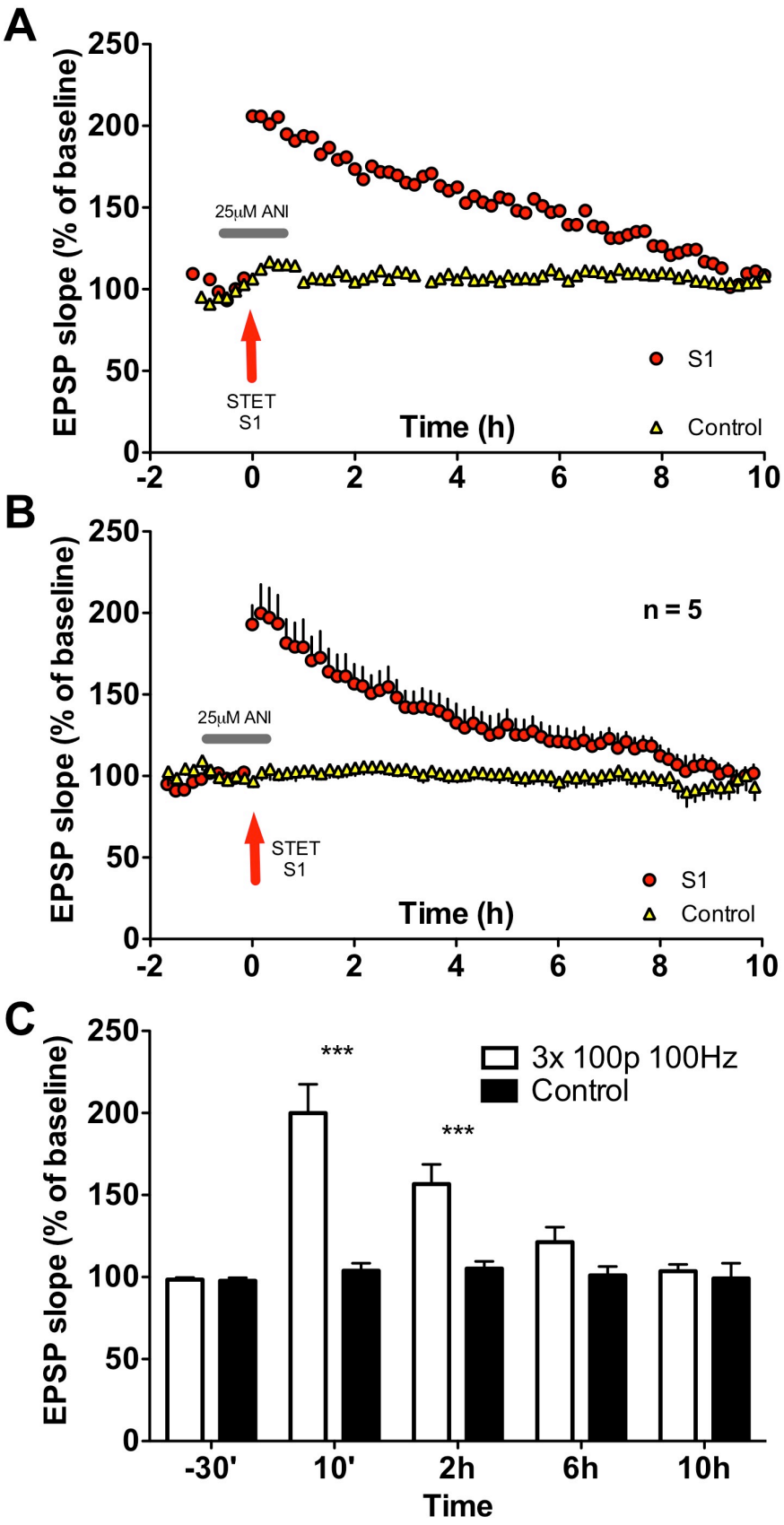


Figure 3.5 25 μM Anisomycin blocks the late-phase of LTP.

A. An individual experiments showing a representative example of the induction of L-LTP under the influence of 25 μM Anisomycin.

B. L-LTP is not maintained when a 'strong tetanus' is given in the presence of 25 μM of the protein synthesis blocker anisomycin. The potentiation declined to baseline after 10 h (S1 vs. baseline; $t = 0.15$, $p > 0.05$) (n = 5).

C. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed ($F = 41.73$, $p < 0.001$) after 10 min ($t = 8.02$, $p < 0.001$) and after 2 h ($t = 4.3$, $p < 0.001$) but there was no statistical difference at 6 h ($t = 1.69$, $p > 0.05$) or at 10 h ($t = 0.37$, $p > 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
25μM ANI
STET
S1
Time (h)
S1
Control
-2
0
2
4
6
8
10
B
EPSP slope (% of baseline)
n = 5
25μM ANI
STET
S1
Time (h)
S1
Control
C
EPSP slope (% of baseline)
***
***
3x 100p 100Hz
Control
-30'
10'
2h
6h
10h
Time

3.3.4 The induction of LTP is dependent upon the activation of NMDARs.

The induction of LTP has been shown to require the entry of calcium via the activation of NMDAR (Collingridge et al., 1983; Davies and Collingridge, 1989). The signalling pathways involved in the synthesis of the PRPs necessary for the maintenance of LTP are then dependent upon the activation of NMDAR (O'Carroll and Morris, 2004). There are however, ways of inducing changes in synaptic plasticity that do not seem to require the activation of NMDAR and with calcium entering through VGCC or CICR instead (Emptage et al., 1999; Sabatini et al., 2001). These NMDAR-independent forms of potentiation rely on increases in cytoplasmic (Ca^{2+}) that are not limited to the dendritic spines. The evidence points towards NMDAR-driven calcium influx as the mechanism responsible for LTP induction (Yuste et al., 1999; Kovalchuk et al., 2000). To make sure that the LTP induction protocols used in these experiments rely on Ca^{2+} entry through NMDAR, the NMDAR blocker D-AP5 was applied and the 'strong tetanus' (3x 100p 100Hz 10 min apart) was delivered. This strong stimulation failed to induce any sort of potentiation under the presence of APV ($t = 0.47$, $p > 0.05$) (Figure 3.7). After the APV was washed out, and one hour after the previous tetanus, another strong stimulation was delivered to confirm the reversibility of the NMDAR block and under these conditions, L-LTP could be induced ($t = 2.89$, $p < 0.05$) (Figure 3.7).

This experiment supports the NMDAR dependency of the 3x (100p 100Hz) stimulation protocol.

Figure 3.6 Representative example of fEPSP waveforms from an individual experiment with high frequency stimulation under the presence of 25 μM D-AP5.

Individual fEPSPs from the tetanus (S1) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). Theses waveforms are taken from the individual experiment shown in Figure 3.7

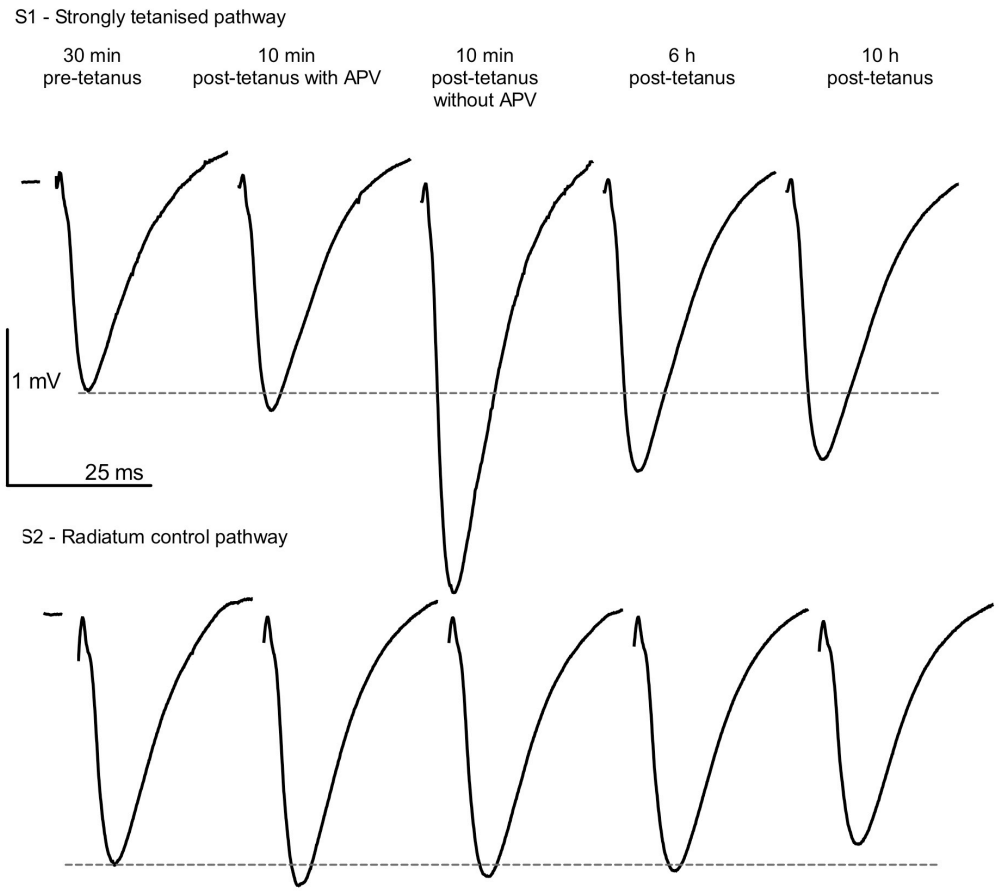
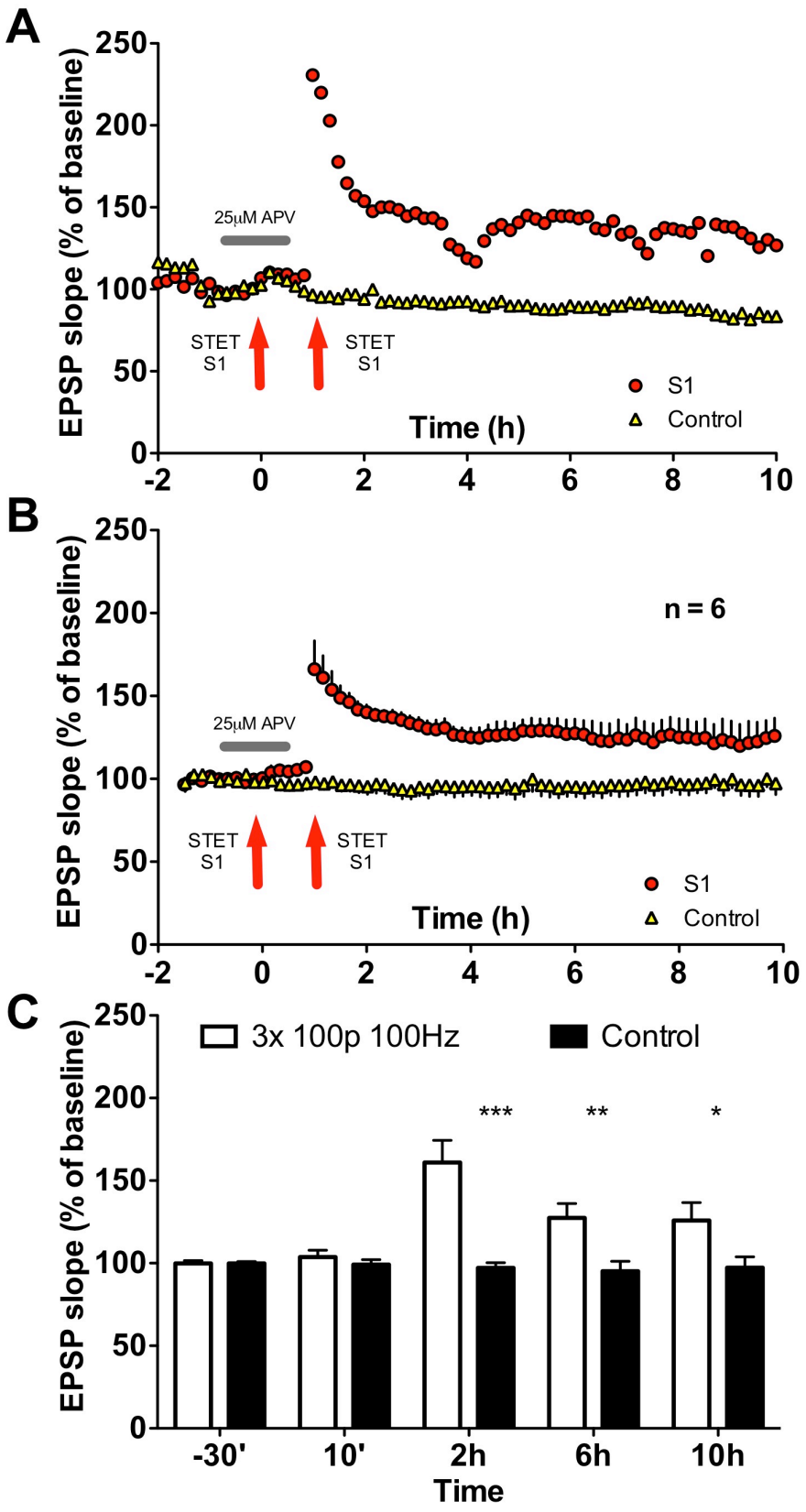


Figure 3.7 25 μM D-AP5 blocks the induction of LTP.

A. An individual experiments showing a representative example of the induction of L-LTP under the influence of 25 μM D-AP5.

B. L-LTP is not induced when a 'strong tetanus' is given in the presence of 25 μM of the NMDAR antagonist D-AP5. The strong tetanization delivered under the presence of D-AP5 failed to induce any type of potentiation (S1 vs. baseline at 30 min post-tetanus; $t = 0.47$, $p > 0.05$) ($n = 6$). However, after washout of the D-AP5, L-LTP could be induced as usual and detected after 10 h (S1 vs. baseline; $t = 2.89$, $p < 0.05$).

C. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed ($F = 34.23$, $p < 0.001$) after the induction of LTP without D-AP5 2 h into the experiment ($t = 6.45$, $p < 0.001$) and this potentiation lasted at least 9 more hours ($t = 2.89$; $p < 0.05$). However, with 25μM D-AP5, the same tetanization protocol failed to induce any potentiation ($t = 0.47$, $p > 0.05$.**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
25μM APV
STET
S1
STET
S1
S1
Control
Time (h)
-2
0
2
4
6
8
10
B
EPSP slope (% of baseline)
n = 6
25μM APV
STET
S1
STET
S1
S1
Control
Time (h)
C
EPSP slope (% of baseline)
3x 100p 100Hz
Control
***
**
*
-30'
10'
2h
6h
10h
Time

3.4 Discussion

In this chapter results have been presented from experiments which demonstrate a reliable method of obtaining NMDAR and protein synthesis dependent L-LTP in the CA1 region of the hippocampus. The 100p 100 Hz tetanisation protocol was capable of eliciting a long lasting change in the synaptic efficacy between CA3 Schaffer collateral axons and CA1 synapses. This potentiation of the synaptic response did last for at least ten hours showing the stability required to be considered late long-term potentiation (Figs 3.2 & 3.3).

The L-LTP induced in this way required the synthesis of new PRPs as it is shown by its sensitivity to the presence of Anisomycin (Figs 3.4 & 3.5). When 25 μM Anisomycin was present at the time of induction the resulting LTP succeeded in expressing an early-phase lasting around 4 hours but then the synaptic responses returned to pre-tetanisation levels. There was no difference between the control pathway and the stimulated pathway 10 h after the tetanisation. This experiment reveals a protein-synthesis dependent phase in the LTP elicited with 3 trains of 100p 100Hz delivered every 10 min.

The 'strong tetanus' induces L-LTP in an NMDAR-dependent manner as seen by the reversible blockade of synaptic potentiation by the NMDAR antagonist D-AP5 (Figs 3.6 & 3.7)

Chapter 4: Induction of early LTP (E-LTP)

4.1 Introduction

In Chapter 3, it was shown that a protein synthesis dependent L-LTP can be induced with multiple trains of high frequency stimulation. This chapter describes experiments that lead to the induction of a shorter lasting early-LTP (E-LTP). As explained in chapter 1 and discussed in chapter 11, the STC hypothesis predicts the presence of a protein-synthesis independent mechanism acting locally at the potentiated synapses (the tag). The aim of the experiments in this chapter was to find a stimulation protocol capable of eliciting a decaying form of long-term potentiation that could later be used as 'weak stimulation' in experiments of heterosynaptic plasticity.

4.1.1 Differences between E-LTP and L-LTP

The distinction between L-LTP and E-LTP is mainly based on the dependency of the former on protein synthesis while E-LTP is a phase that does not require the transcription of new PRPs. The literature identifies E-LTP as a form of potentiation of the synaptic response that returns to pre-stimulation levels between 2 and 6 hours (Kelleher et al., 2004b). Electrophysiologically, E-LTP is obtained by weaker synaptic stimulation. In the CA1 region of the hippocampus where all these forms of plasticity are studied there seems to be a threshold of stimulation above which the machinery leading to the synthesis of new PRPs is engaged. If the stimulation is kept below the threshold, LTP can still be induced and synaptic modifications take place capable of sustaining an increased response to presynaptic stimulation. A weak stimulation that does not reach the threshold for synthesis of PRPs should trigger a temporary change.

4.1.2 Strength vs. persistence

It is worth emphasizing here the difference between strength and persistence when dealing with LTP and memories. Measurements at certain delays are used to study

the maintenance of a memory or of a change in synaptic efficacy. In this way, a memory can be enhanced in such a way that while under normal circumstances the subject would perform poorly after a given delay (i.e. 24 h), due to some manipulation, the performance is significantly improved after such a delay. The question remains, however, as to whether the manipulation helped the maintenance of the memory or whether the manipulation increased the strength of the memory from the time of encoding.

An analogous measurement can be done for the potentiation of a synaptic response by measuring its level after a given delay. Manipulations that allow LTP to be present after a delay (i.e. 10h) can act by stabilizing the maintenance of a given potentiation or by facilitating the initial expression of LTP in such a way that even under a similar decay rate, there is enough potentiation for as long as measurements are conducted. To distinguish from a gain in initial strength and a stabilization of synaptic change, we compared the levels of potentiation present 2 h post-tetanus with those remaining after 10 h. LTP was considered stabilized if no significant difference was found between the potentiation levels at those two time points (also described in chapter 2 section 2.5).

4.2 Methods

Similar procedures for the preparation and incubation of slices were used as described in Chapters 2 and 3.

4.2.1 Preparation of slices and recording set-up.

Artificial cerebrospinal fluid (aCSF) was prepared with the following concentrations: NaCl 124 mM, KCl 3.7 mM, KH_2PO_4 1.2 mM, $MgSO_4(7H_20)$ 1.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.6 mM, D-glucose 10 mM (pH = 7.4). 7 to 8 week old male Wistar rat was anaesthetized with halothane or isofluorane, before the brain was removed and sectioned as described previously (Leutgeb et al., 2003). 400 μm thick brain slices were sectioned with a Vibratome (Campden Instruments Integraslice 7550 PSDS) using stainless steel blades (Campdem Instruments 7550/1/SS). The brain slices were kept in a resting chamber with oxygenated aCSF for less than 5 min before being transferred into the experimental chamber. Three monopolar stainless steel stimulating electrodes (A-M systems) and the one stainless steel recording electrode were positioned as in Figure 2.1. The rate of stimulation provided 1 data point per stimulated channel every 2.5 min. For three pathway experiments this means that one channel is stimulated every 50 seconds (0.02 Hz).

4.2.2 Tetanus protocol.

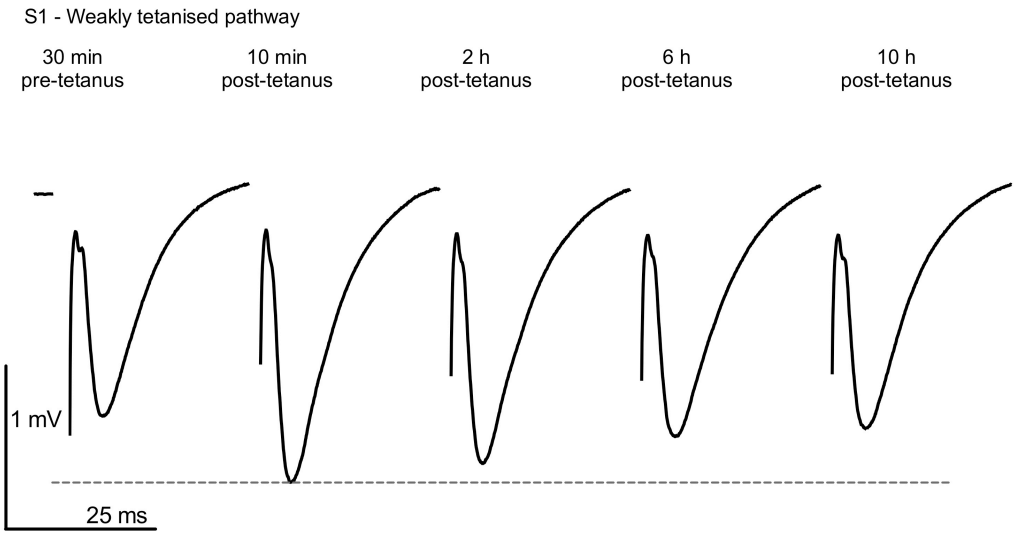
For the induction of E-LTP, the tetanus protocol consisted of 20 pulses delivered in 4 trains of 5 pulses each (biphasic pulses with 100 μs per half-wave). The 4 trains were 200 msec apart, or at 5 Hz, which fits the frequency range of theta rhythms. Experiments in which the drift in the control non-tetanised pathway was > 30% in either direction over the 10 h post-tetanus period were excluded from analysis. Recordings were made for at least 10 h post-tetanus.

4.3 Results

The Input Output curves and the selection of the test stimulation frequencies were done as described in chapters 2 and 3. Representative field EPSPs of the pathway of interest are shown from an individual experiment in Fig 4.1 Control traces are similar to those depicted in the Chapter 2 Fig. 6

Figure 4.1 representative examples of fEPSP waveforms from an individual experiment with 20 pulses TBS.

Individual fEPSPs from the tetanus (S1) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 4.2A



4.3.1 E-LTP is induced by 20 pulses delivered in a theta-burst fashion.

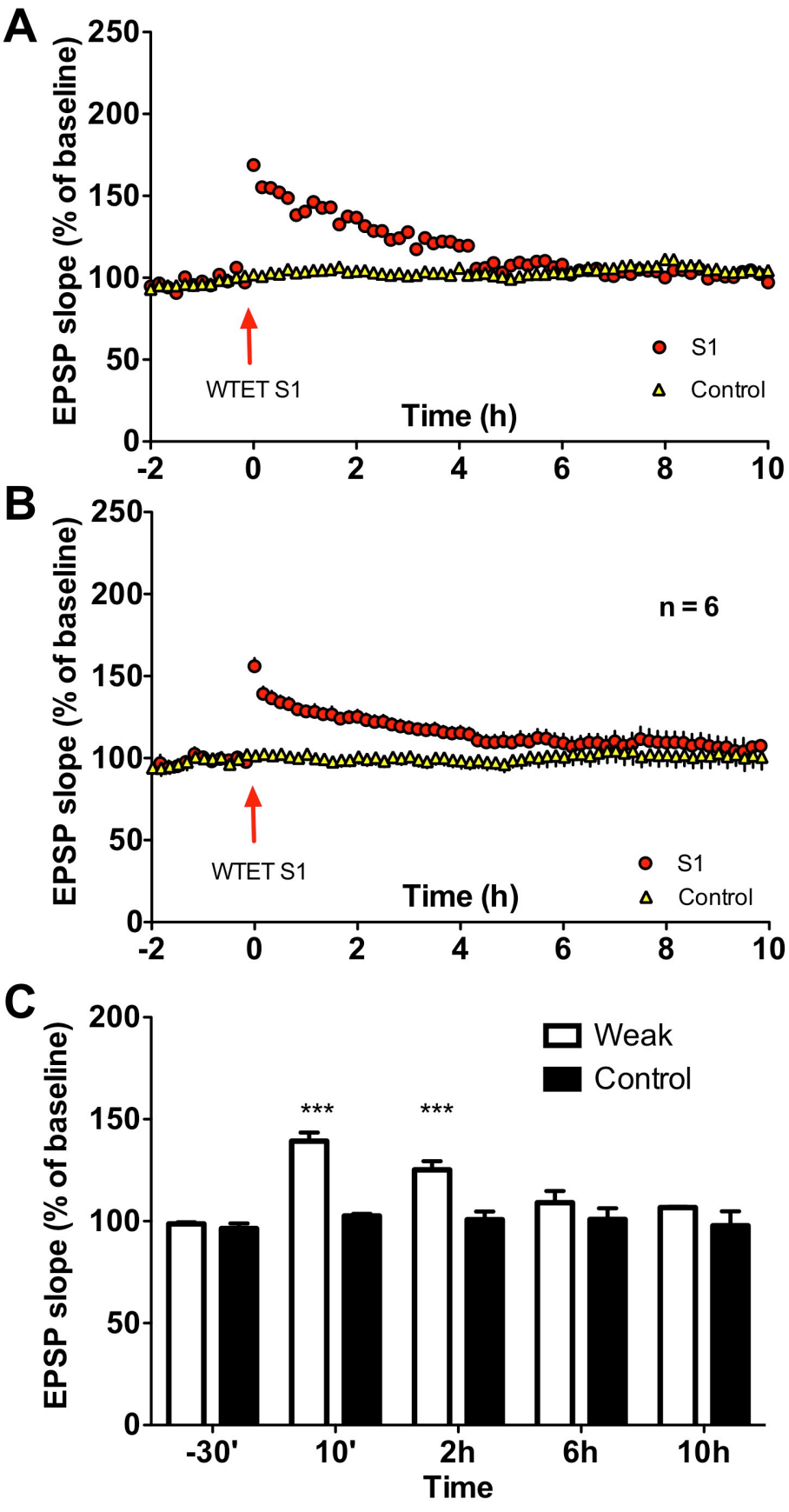
A potentiation of the synaptic response lasting more than 2 h but eventually decaying to baseline was obtained when 20 pulses delivered in a Theta Burst rhythm was used. Compared to the control pathway, S1 showed a potentiation of its synaptic response to test stimulation at 2 h ($t = 4.3$, $p < 0.01$) but this potentiation had declined already after 6 h ($t = 1.02$, $p > 0.05$) and was absent after 10 h ($t = 0.35$, $p > 0.05$). There is a decline in synaptic strength when comparing the level of potentiation present at 2 h (125% ± 4.1) with that remaining at 10 h (101% ± 7.8) ($t = 2.9$, $p < 0.05$). A statistically significant difference confirms the failure of those synapses to maintain the synaptic changes.

Figure 4.2 Induction of E-LTP with 20p TBS.

A. An individual experiments showing a representative example of the induction of E-LTP.

B. Grouped data for experiments in which S1 received 20p TBS stimulation. This stimulation lead to a potentiation that was present at 2 h but that declined to pre-tetanus levels after 10 hours (S1 vs. baseline; $t = 0.56$, $p > 0.05$) ($n = 6$).

C. Statistical comparisons were made between the tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed after 10 min ($t = 6.19$, $p < 0.001$) and after 2 h ($t = 4.13$, $p < 0.001$) but there was no statistical difference at 6 h ($t = 1.39$, $p > 0.05$) or at 10 h ($t = 1.52$, $p > 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
WTET S1
Time (h)
S1
Control
B
EPSP slope (% of baseline)
n = 6
WTET S1
Time (h)
S1
Control
C
EPSP slope (% of baseline)
Weak
Control
***
***
-30'
10'
2h
6h
10h
Time

4.4 Discussion

In this chapter results have been presented from experiments that demonstrate a reliable method of obtaining E-LTP in CA1. The 20p TBS tetanisation protocol was capable of eliciting a change in the synaptic efficacy between CA3 Schaffer collateral axons and CA1 synapses that lasted between 4 and 6 h. The E-LTP inducing protocol described here (20 p TBS) will be used in the following chapters and referred to as 'weak tetanus'. Different laboratories and researches have developed other protocols to reach a similar protein-synthesis independent E-LTP. Some researchers have resorted to a single train of 100p 100Hz while other had to go as low as 11 pulses 100Hz in order to obtain a decaying potentiation (O'Carroll and Morris, 2004). Besides the health and the stability of the preparation (described in chapter 2 Methods), one of the sources of variability is the distinct test stimulation rate between laboratories. The slow rate used in these experiments (0.006 Hz) may be responsible for the slow decay of potentiation (see methods chapter 2). In this set of experiments, 20 pulses delivered in a theta-burst fashion have been shown to beunable to induce synaptic potentiation that could be maintained for 10 h. In the following chapter, this LTP-induction protocol will be used to investigate a form of heterosynaptic plasticity explained by the synaptic tagging and capture hypothesis.

Chapter 5: Synaptic tagging replication

5.1 Introduction

As seen in Chapter 3 and 4, long-lasting changes in the weight of synaptic responses can be elicited with particular types of high-frequency stimulation. The duration of these changes is of scientific interest because the synaptic plasticity and memory hypothesis proposes a link between the maintenance of synaptic changes and the consolidation memories. What factors determine whether a potentiation present 2 h after induction will remain after 10 h or whether it will return to pre-stimulation levels? If new proteins are necessary for the maintenance of L-LTP (as seen in chapter 3), where are these proteins synthesized within the neuron and how are they directed towards their sites of action? What are the interactions between the thousands of synapses present in the dendritic tree? These questions can be tackled with the use of independent stimulation pathways capable of engaging different sets of synapses convergent onto the same neurons.

5.1.1 The Synaptic Tagging and Capture Hypothesis

The synaptic-tagging and capture (STC) hypothesis of L-LTP (Frey and Morris, 1997) asserts that the maintenance of LTP requires the local 'tagging' of synapses at the moment of induction, that such tags capture diffusely transported PRPs synthesised in the soma or local dendritic domains, and that tag-PRP interactions takes place to stabilize potentiated synapses. In other words, L-LTP requires two parallel processes, the synthesis of new proteins distributed around the dendrites and the setting of a local tag at stimulated synapses which needs to capture these PRPs in order to stabilize LTP. The identity and properties of the tag and its interactions have been studied in order to better understand the molecular basis of learning and memory (Martin and Kosik, 2002; Fonseca et al., 2004; Navakkode et al., 2004; Sajikumar and Frey, 2004a, b; Zhong and Zucker, 2004; Navakkode et al., 2005; Sajikumar et al., 2005c; Young and Nguyen, 2005; Alarcon et al., 2006; Young et al., 2006; Lopez-Rojas et al., 2007; Reymann and Frey, 2007; Sajikumar et al., 2007; Viosca et al., 2007; Okada et al., 2009).

5.1.2 The benefit of a third control pathway

Traditionally, STC hypothesis has been tested by using two independent inputs into the same neuronal population (Frey and Morris, 1997, 1998a, b; Sajikumar and Frey, 2004a; Sajikumar et al., 2005c; Alarcon et al., 2006; Young et al., 2006; Sajikumar et al., 2007). As shown in the methods chapter 2, slight chances in the humidity, temperature and pH can lead to changes in the overall excitability of the slice preparation. As slow as this changes may be, the baseline recordings representing pre-stimulation levels of synaptic strength may become misleading without a live reference of overall cellular excitability. In two pathway experiments, once the two pathways have received some tetanic stimulation, there will be no point of reference available to the current state of synaptic excitability in the preparation. A third non-tetanised pathway helps to avoid the risks of misleading assumptions as to the potentiated state of a synaptic pathway many hours after the induction of synaptic plasticity. In this way there is always a point of reference to which comparison of potentiated synaptic states can be made. Across all the experiments described in this and future chapters of this thesis, the third control pathway is obtained by measuring the synaptic responses to *stratum oriens* axonal stimulation. These synaptic responses are measured in the *stratum radiatum* and therefore are detected as positive deviations in voltage.

5.1.3 The present experiments

The present experiments deal with one form of heterosynaptic modulation of LTP that may be explained by the STC hypothesis. As described in chapter 1 the outcome of a change in synaptic efficacy can be determined during the induction of that change (i.e. by blockade of NMDARs). Interestingly, the persistence of long term changes in the strength of one set of synapses can be influenced by events happening to another set of synapses. This modulation does not need to occur during the induction of a synaptic change and depends on the synthesis of new proteins. The immediate goal of the experiments in this chapter was to reproduce the phenomenon whereby an L-LTP inducing stimulus delivered to one set of synapses allows another set of synapses onto the same neuronal population to maintain their synaptic potentiation after a weaker, E-LTP inducing tetanisation.

The replication of the STC experiments was attempted in both ‘Strong before Weak’ and ‘Weak before Strong’ protocols. In the original experiments, the order of stimulation did not interfere with the rescue of E-LTP into L-LTP in the weakly tetanised pathway (Frey and Morris, 1998b).

5.2 Methods

Similar procedures for the preparation and incubation of slices were used as described in Chapters 2. 3 and 4.

5.2.1 Preparation of slices, and recording set-up

Artificial cerebrospinal fluid (aCSF) was prepared with the following concentrations: NaCl 124 mM, KCl 3.7 mM, KH_2PO_4 1.2 mM, $MgSO_4(7H_20)$ 1.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.6 mM, D-glucose 10 mM (pH = 7.4). 7 to 8 week old male Wistar rat was anaesthetized with halothane or isofluorane, before the brain was removed and sectioned as described previously (Leutgeb et al., 2003). 400μm thick brain slices were sectioned with a Vibratome (Campden Instruments Integraslice 7550 PSDS) using stainless steel blades (Campdem Instruments 7550/1/SS). The brain slices were kept in a resting chamber with oxygenated aCSF for less than 5 min before being transferred into the experimental chamber. Three monopolar stainless steel stimulating electrodes (A-M systems) and the one stainless steel recording electrode were positioned as in Figure 2.1. The rate of stimulation provided 1 data point per stimulated channel every 2.5 min. For three pathway experiments this means that one channel is stimulated every 50 seconds (0.02 Hz)4.

5.2.2 Tetanus protocol

L-LTP was obtained with the strong stimulation delivered using the protocol described in chapter 3 and consisted of 3 trains of 100 biphasic pulses at 100Hz delivered 10 min apart. E-LTP was elicited using the weak stimulation protocol described in chapter 4 and consisted of 20 biphasic pulses delivered in bursts of 5 pulses 100 Hz, 200 ms apart.

5.3 Results

The Input Output curves and the selection of the test stimulation frequencies were done as described in chapter 2 and 3. Representative field EPSPs of the pathway of interest are shown from an individual experiment in Fig 5.1 Control traces are similar to those depicted in the Chapter 2 Fig. 2.6.

5.3.1 Rescue of decremental LTP by a prior 'strong' tetanus.

Weak stimulation (Chapter 4) given to one set of synapses is capable of eliciting L-LTP if previously, another set of synapses on the same cell population received strong stimulation (Chapter 3). When a strong tetanus protocol was given to one pathway and, 20 min later, a weak tetanus was applied to a second independent, but convergent pathway L-LTP was observed on both pathways lasting > 14 h (Fig. 5.2A Single experiment). The group data (Fig. 5.2B) of a series of experiments revealed that the weakly tetanised pathway maintained its potentiated state for at least 10 h, as shown by comparing the potentiation of the weakly tetanized pathway with that of the third control pathway ($t = 3.4$, $p < 0.01$). There was also stability over the 2 to 10 h post-tetanization time-period (S2 at 2 vs. 10 h; $t = 2.2$; $p > 0.05$). This potentiation was also different from that of weakly tetanized pathways in slices that did not receive any strong stimulation (Compare S2 in Fig 5.2B with S1 in Fig 4.2B at 10 h; $t = 3.4$, $p < 0.05$).

Figure 5.1 Representative fEPSPs waveforms from an individual 'Strong before Weak' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.2A

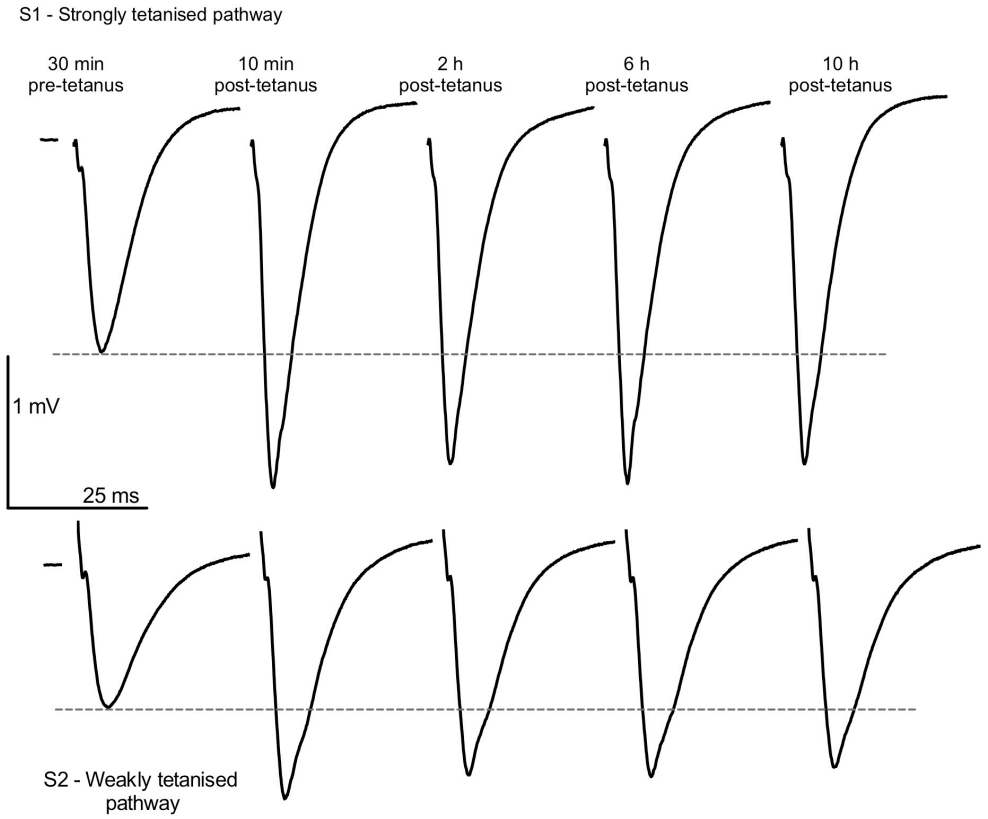
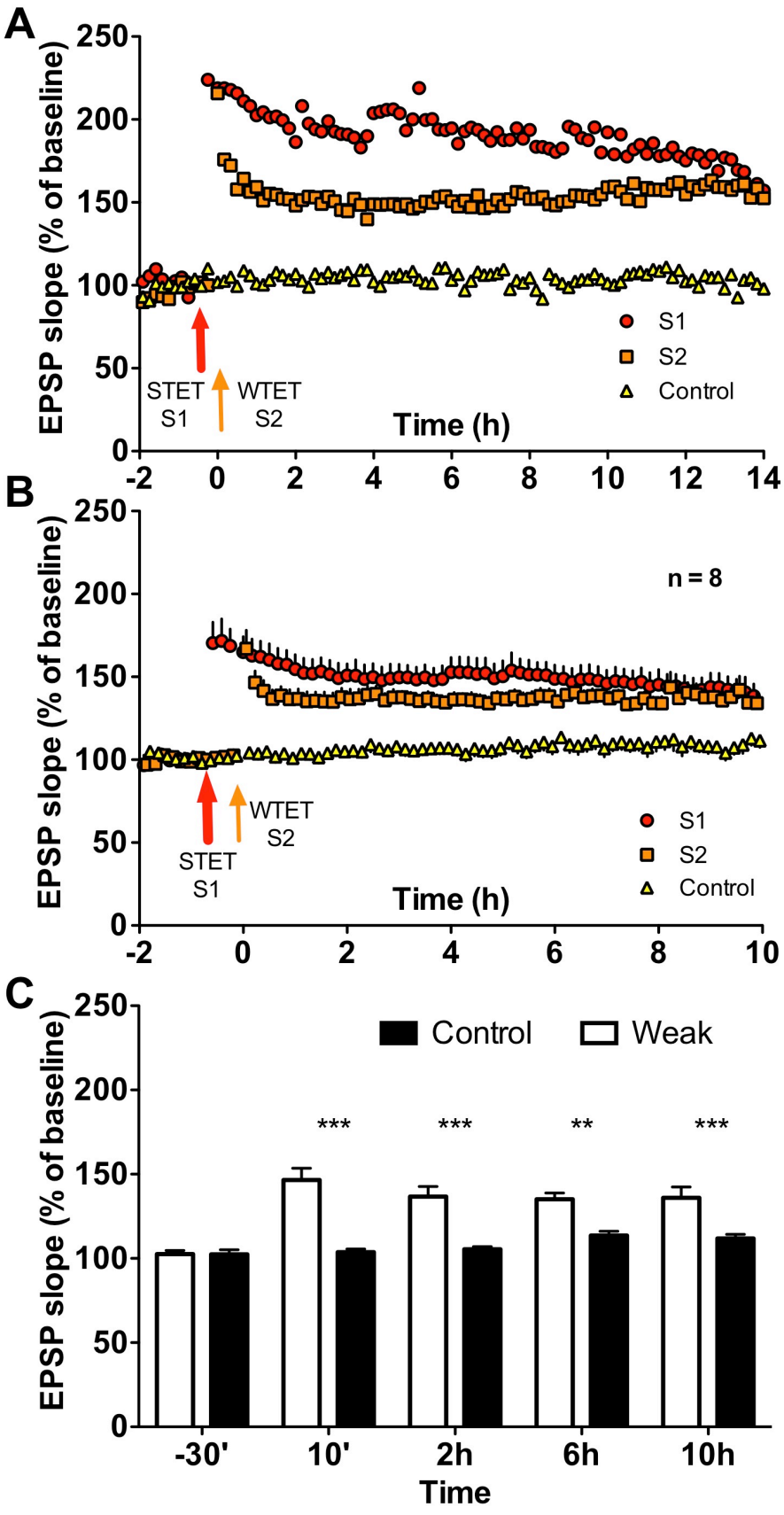


Figure 5.2 Synaptic tagging: Rescue of decremental LTP by prior strong heterosynaptic stimulation.

A. An individual experiments showing a representative example of the rescue of L-LTP in a weakly tetanised pathway by prior strong tetanization of another pathway.

B. Grouped data for experiments in which S2 received 20p TBS stimulation and the resulting potentiation was maintained for over 10 h when compared to the control pathway ($t = 3.4$, $P < 0.01$) ($n = 8$).

C. Statistical comparisons were made between the weakly-tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 7.29$, $p < 0.001$), after 2 h ($t = 5.37$, $p < 0.001$) after 6 h ($t = 3.65$, $p < 0.01$) and after 10 h ($t = 4.18$, $p < 0.001$).**

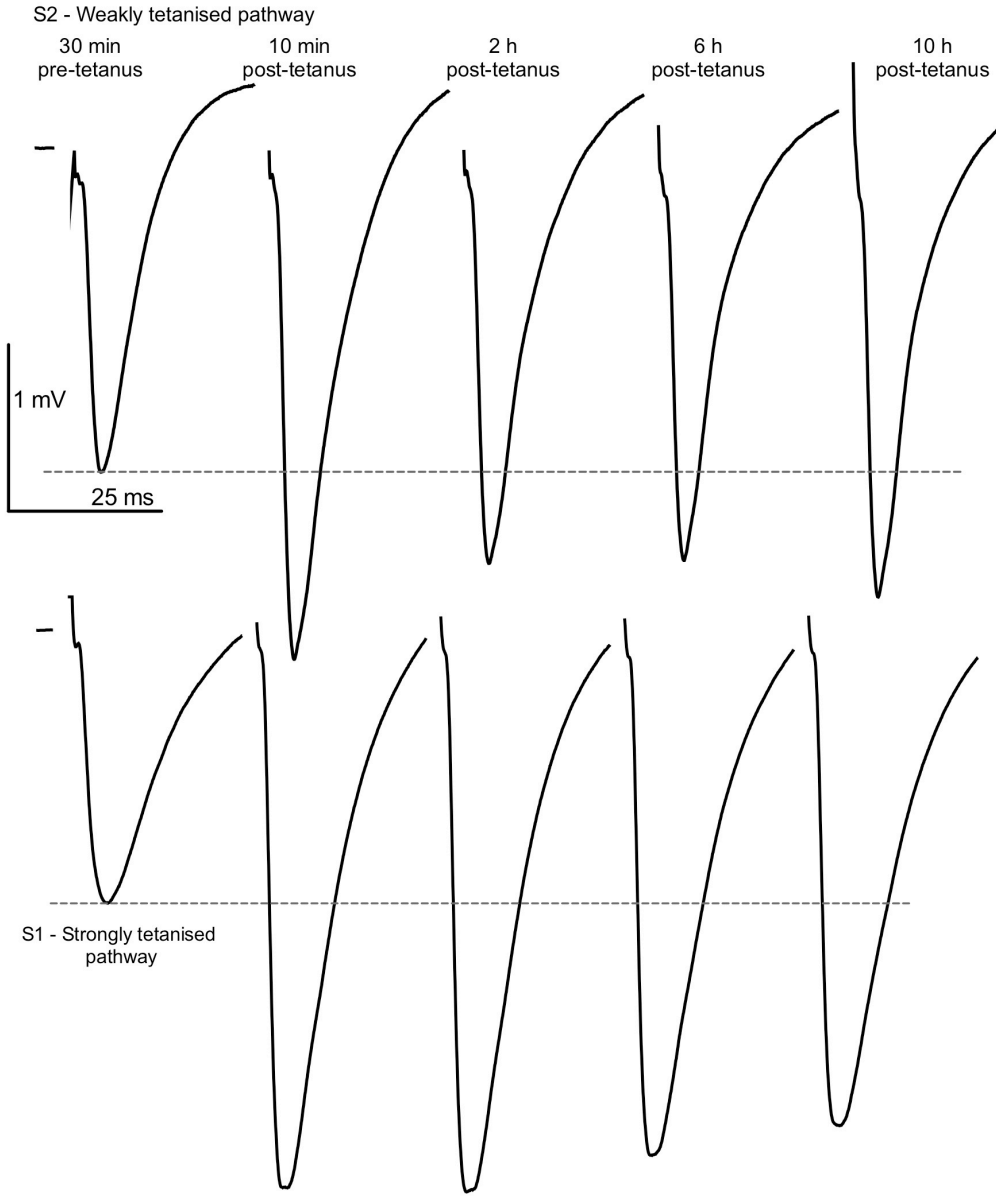

A
EPSP slope (% of baseline)
250
200
150
100
50
0
STET
S1
WTET
S2
Time (h)
-2
0
2
4
6
8
10
12
14
S1
S2
Control
B
n = 8
WTET
S2
STET
S1
C
Control
Weak
***
***
**
***
-30'
10'
2h
6h
10h
Time

5.3.2 Weak before strong experiments

The rescue of E-LTP into L-LTP can be explained by the STC hypothesis whereby the PRPs necessary for the maintenance of L-LTP are made available by the strong tetanization to one set of synapses and the weakly-tetanised synapses can capture these PRPs due to some mark or tag. To assess whether this heterosynaptic interaction can be obtained independently of the order of stimulation, another set of experiments were carried out where the weak tetanus was delivered before the strong. This protocol also avoids any facilitatory effect of the strong stimulation when given before the weak tetanus. The cellular and synaptic state at the time of induction should be identical both in the weak-alone and in the weak-before-strong protocol. The following figures attempt to assess this prediction and corroborate the order-independence quality of the heterosynaptic mechanisms that the STC hypothesis accounts for.

Figure 5.3 Representative fEPSPs waveforms from an individual 'Weak before Strong' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 5.4A


S2 - Weakly tetanised pathway
30 min
pre-tetanus
10 min
post-tetanus
2 h
post-tetanus
6 h
post-tetanus
10 h
post-tetanus
1 mV
25 ms
S1 - Strongly tetanised
pathway

5.3.3 Weak before Strong results in the transformation of E-LTP into L-LTP in a pathway given brief high frequency stimulation.

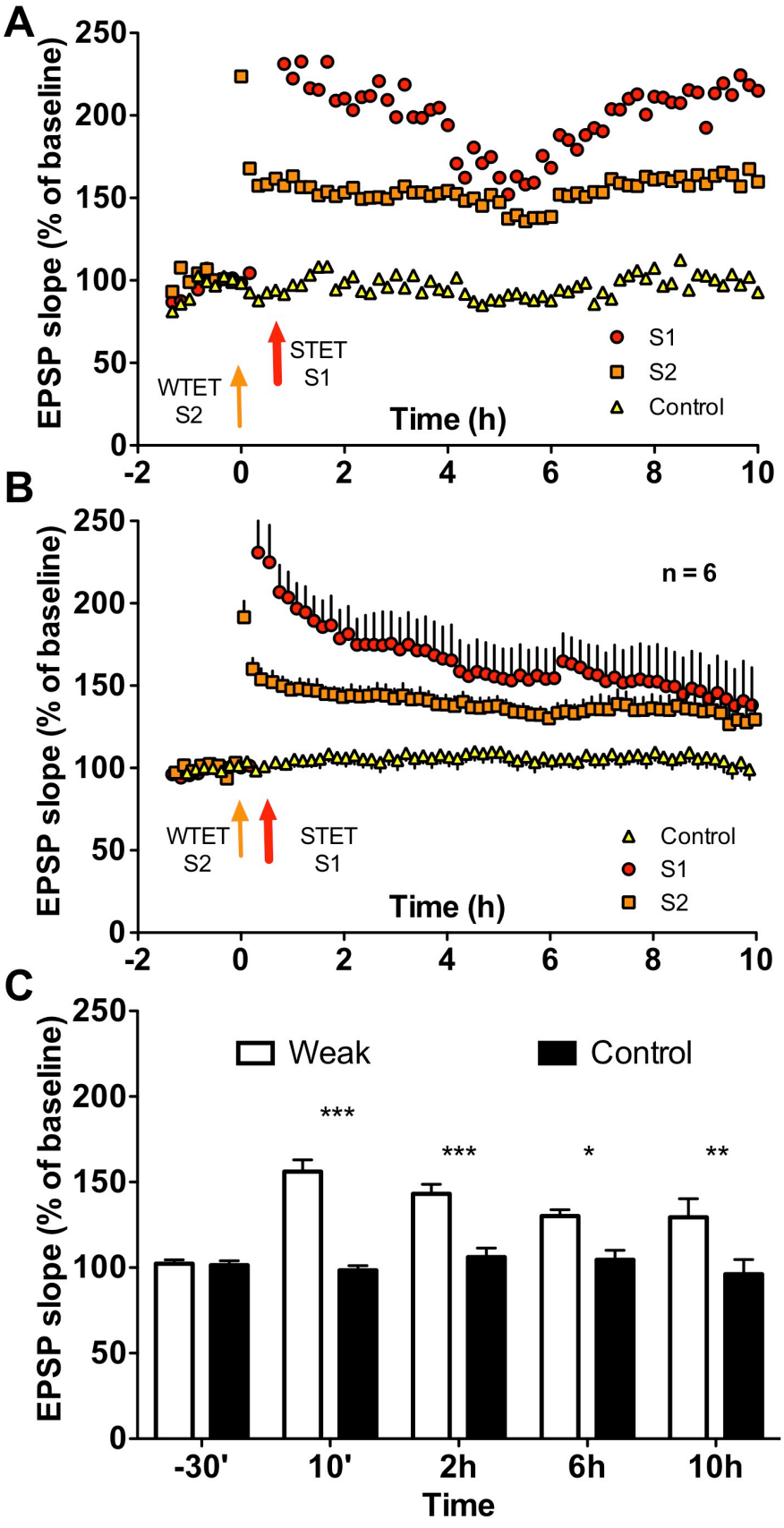
As predicted by the STC hypothesis and revealed originally in 1998 (Frey and Morris, 1998b) synapses that underwent an E-LTP induction protocol can maintain their change in synaptic efficacy and express L-LTP if after the induction another set of synapses convergent on the same neuronal population is strongly stimulated with an L-LTP inducing protocol. In normal circumstances, the weakly stimulated pathway (S2) will express E-LTP but fail to maintain L-LTP (Chapter 4). However, as seen in Figure 5.4, S2 is E-LTP is rescued into L-LTP if another pathway (S1) experiences a strong-tetanization protocol. The implications of these results are discussed later in this chapter.

Figure 5.4 Synaptic tagging: rescue of decremental LTP by subsequent Strong heterosynaptic stimulation.

A. An individual experiments showing a representative example of the rescue of L-LTP in a weakly tetanised pathway (S2) by subsequent strong tetanization of another pathway (S1).

B. Grouped data for experiments in which S2 received 20p TBS stimulation and the resulting potentiation was maintained for over 10 h when compared to the control pathway ($t = 3.99$, $p < 0.01$) ($n = 6$).

C. Statistical comparisons were made between the weakly-tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 6.81$, $p < 0.001$), after 2 h ($t = 4.36$, $p < 0.001$) after 6 h ($t = 3.01$, $p < 0.05$) and after 10 h ($t = 3.93$, $p < 0.01$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
Time (h)
WTET
S2
STET
S1
S1
S2
Control
B
n = 6
WTET
S2
STET
S1
Control
S1
S2
C
Weak
Control
***
***
*
**
-30'
10'
2h
6h
10h
Time

5.4 Discussion

The results of the present experiments reproduce the phenomenon described in 1997 (Frey and Morris, 1997) and explained under the synaptic capture and tagging hypothesis (STC). In summary, the fate of a change in synaptic efficacy at one set of synapses can be determined by the experiences in an independent set of synapses onto the same cell (convergent inputs) (Abraham, 2008). The specific mechanisms that allow this interaction involve the dissociation between a local change at the synapses of interest and the far-reaching production of mobile proteins required to stabilize those local changes. Interestingly, there is a threshold over which both processes are initiated and stimulation that does not reach that threshold sets only the local changes in motion. The experiments described in this chapter combine two strengths of stimulation and deliver them to different sets of synapses onto the same neuronal population within a time window. The resulting data reveals a phenomenon by which those synapses stimulated below the threshold required to maintain their potentiation now succeed in showing L-LTP. The strong stimulation in one set of synapses does not in itself change the synaptic strength in the other set but something happens at the cellular level that allows the weakly stimulated synapses to stabilize their potentiation. The original STC experiments revealed that plasticity related proteins are the ingredient made available to synapses by the strong stimulation (Frey and Morris, 1997). Those experimental results were explained by the synaptic tagging and capture hypothesis whereby local synapses are tagged after stimulation and, if available, are then capable of capturing the plasticity related proteins. (Frey and Morris, 1998a).

This chapter tested the STC hypothesis by showing that E-LTP can be rescued into L-LTP by combining the weak tetanization of one set of synapses with the strong stimulation of another convergent pathway.

As described in chapter 1, the molecular mechanisms behind the STC hypothesis can be investigated by carefully combining specific antagonists with particular stimulation protocols. The next two chapters will present some recent advances in our knowledge of these molecular mechanisms.

In addition, the implications of the STC hypothesis for behavioural memories will be tested in chapters 8 to 10.

Chapter 6: Role of the calcium-calmodulin kinase II in protein synthesis-dependent long-term potentiation.

6.1 Introduction

The STC hypothesis points to two requirements for long term changes in synaptic efficacy: the setting of tags at stimulated synapses and the availability of plasticity related proteins that are captured by the tags and thereby set in train mechanisms responsible for stabilisation of potentiation (Frey et al., 1988; Martin et al., 1997; Martin and Kosik, 2002; Scharf et al., 2002; Fonseca et al., 2004; Karpova et al., 2006; Reymann and Frey, 2007). Amidst the potentially numerous molecular players and complex interactions involved in these processes, one can predict that some molecules (or their activation) may be required for both the setting of the tag and the availability of PRPs (e.g. activation of N-methyl-D-aspartate receptors), whereas other molecules (or molecular states, such as phosphorylation) are necessary for tag setting and others for PRP synthesis and/or availability (see chapter 1 section 1.4.4).

6.1.1 CaMKII as a candidate for a tag-specific role

The present chapter explores the possible role of Ca^{2+}/calmodulin-dependent kinase II (CaMKII) in STC. This broad range kinase regulates many neuronal functions (Erondu and Kennedy, 1985; Braun and Schulman, 1995; Yamauchi, 2005). In the CA3 to CA1 pathway of the hippocampus, CaMKII activation by calcium calmodulin (Ca^{2+}/CaM) is necessary at the time of LTP induction (Malenka et al., 1989), but not during LTP maintenance (Malinow et al., 1989; Otmakhov et al., 1997; Chen et al., 2001). This is possible due to the fact that the 12 subunits that form this holoenzyme have the ability to autophosphorylate each other in the presence of calcium-calmodulin (Kuret and Schulman, 1984; Yamauchi and Fujisawa, 1985). Firstly, the required binding of calmodulin in the presence of Ca^{2+} disrupts the auto inhibitory domain of CaMKII. Then, the phosphorylation of threonine 286 (Thr^{286}) increases the affinity for calmodulin creating a state where calmodulin is trapped by CaMKII even after the end of a calcium transient (Meyer et al., 1992). It has been proposed that this property may enable CaMKII to act as a

switch capable for maintaining changes in synapse efficacy (Lisman and Goldring, 1988; Miller et al., 2005).

This switch quality could account for tag setting or for the capture of newly synthesized PRPs. However, in addition to the functions attributed to CaMKII in the dendritic spine, this kinase is phosphorylated in the soma after tetanic stimulation and engages transcription factors that regulate the synthesis of new PRPs (Ouyang et al., 1997). Is CaMKII a molecule necessary for the setting of the tag only, or is its activation necessary for the availability of PRPs? (Fig. 1.1 & 6.1)

Our goal was to test whether KN-93, a CaMKII inhibitor, would block long-term potentiation in the CA1 region of the hippocampus of acute brain slices by interfering specifically with the molecular switch property of the enzyme (one putative mechanism for tag-setting) and/or would also interfere with the availability of PRPs needed to maintain LTP.

6.1.2 Information obtained by applying inhibitors during weak or strong stimulation

As described in chapter 1 (section 1.4.4), in the processes leading to the expression and stabilization of changes in synaptic efficacy, one can theoretically assign some potential roles to different molecules known to be involved in LTP.

Briefly, in the 'PRP block' experiment (Fig 1.3) a reversible inhibitor capable of blocking LTP is used on one strongly tetanized pathway and after wash-out of the drug, another strong tetanus is applied to a second pathway. The critical variable in this type of experiments is what happens to the synapses strongly tetanized under the influence of the inhibitor. If they succeed in maintaining L-LTP, we have to assume that the synapses were capable of making use of PRPs (introduced by the second stimulation). This result would mean that under the effect of the inhibitor capable of blocking L-LTP in control experiments, the setting of local tags remained functional so as to allow capture once the PRPs are brought about by a second stimulation. The alternative outcome of the 'PRP block' experiment is that the pathway stimulated under the influence of the drug of interest fails to show L-LTP. In this case, the PRPs are available to the other stimulated pathway as it succeeds in maintaining L-LTP. One possible conclusion from this outcome is that the inhibitor prevented the setting

or the action of the tag at the synapses where it acted. What this outcome does not clarify is whether the block of the tag is specific or whether the inhibitor also blocks the pathways that enable the synthesis of PRPs.

In the 'tag block experiment' (Fig 1.4) an inhibitor of LTP is present during the strong tetanization of one pathway and later, after drug washout, an E-LTP-inducing weak tetanus is delivered heterosynaptically. If in this experiment, the weakly tetanized synapses succeed in showing L-LTP while the strongly tetanized decay to baseline levels of synaptic efficacy, the drug should have been interfering with the tag but not with availability of the products of the *de novo* protein synthesis. In other words, the fact that the weakly stimulated synapses maintain L-LTP requires their successful use of PRPs. These PRPs could only have been produced by the strong tetanus even though it was delivered to the first pathway under the influence of our theoretical inhibitor. Because the strongly tetanized synapses fail to show L-LTP, even with PRPs available to the other pathway, their synapses must have dysfunctional tags. This result is definitive in pinpointing a tag-specific role to whatever molecule or process the theoretical inhibitor was interfering with.

The combination of 'PRP block' and 'Tag block' experiments should be definitive in determining necessary roles in the sequence of events leading to STC to candidate molecules.

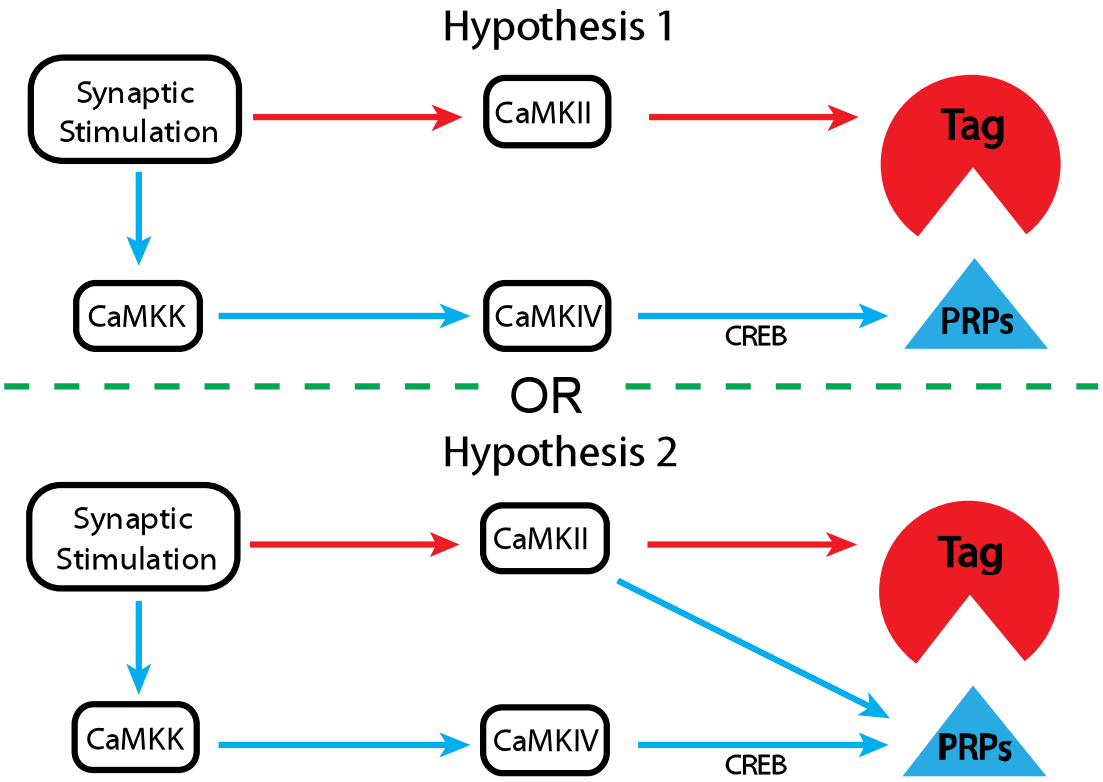
6.1.3 The present experiments

The present study explores the potentially differential roles of Ca^{2+}/calmodulin-dependent kinase II (CaMKII)-dependent pathway in the persistence of LTP. While CaMKII is a kinase that regulates many neuronal functions (Erondu and Kennedy, 1985; Braun and Schulman, 1995; Yamauchi, 2005), various theoretical ideas and experimental lines of evidence suggest that its activation at synapses of the CA3 to CA1 pathway of the hippocampus is involved in the initial expression of LTP (Lisman and Goldring, 1988; Malenka et al., 1989; Malinow et al., 1989), but there is discussion about the role of CaMKII in LTP maintenance (Malinow et al., 1989; Otmakhov et al., 1997; Bortolotto and Collingridge, 1998; Chen et al., 2001; Sanhueza et al., 2007). Might there also be a separate role for CaMKII in the process of tag setting that is independent of any role in LTP induction or maintenance? The

experiments described in this chapter set out to test whether selective inhibitors of different CaM Kinases are capable of specifically interfering with either the setting of tags or the synthesis of PRPs (Fig. 6.1).

Figure 6.1 Outline of the hypothesis.

Is the necessary role of CaMKII in LTP limited to the setting of a synaptic tag or is CaMKII activity also necessary on the signalling pathway leading to the synthesis of PRPs?



6.2 Methods

Similar procedures for the preparation and incubation of slices were used as described in Chapters 2 and 3.

6.2.1 Preparation of slices, and recording set-up

Artificial cerebrospinal fluid (aCSF) was prepared with the following concentrations: NaCl 124 mM, KCl 3.7 mM, KH_2PO_4 1.2 mM, $MgSO_4(7H_20)$ 1.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.6 mM, D-glucose 10 mM (pH = 7.4). 7 to 8 week old male Wistar rat was anaesthetized with halothane or isofluorane, before the brain was removed and sectioned as described previously (Leutgeb et al., 2003). 400μm thick brain slices were sectioned with a Vibratome (Campden Instruments Integraslice 7550 PSDS) using stainless steel blades (Campdem Instruments 7550/1/SS). The brain slices were kept in a resting chamber with oxygenated aCSF for less than 5 min before being transferred into the experimental chamber. Three monopolar stainless steel stimulating electrodes (A-M systems) and the one stainless steel recording electrode were positioned as in Figure 2.1 & 3.1. The rate of stimulation provided 1 data point per stimulated channel every 2.5 min. For three pathway experiments this means that one channel is stimulated every 50 s (0.02 Hz).

6.2.2 Tetanus protocol

L-LTP was obtained with the strong stimulation delivered using the protocol described in chapter 3 and consisted of 3 trains of 100 pulses at 100Hz delivered 10 min apart. E-LTP was elicited using the weak stimulation protocol described in chapter 4 and consisted of 20 pulses delivered in bursts of 5 pulses 100 Hz, 200 ms apart.

6.2.3 Drugs

KN-93 is a potent and selective inhibitor of CaMKII phosphorylation with no significant effects on other kinases. KN-93 competes with calmodulin and also

inhibits the autophosphorylation of both the alpha- and beta-subunits of CaMKII with an IC50 of 0.37 μM (Sumi et al., 1991). The mechanism of action of KN-93, i.e. the competition with calmodulin binding, allows this drug to have an effect when present during the induction of LTP but not during the maintenance of an already potentiated synapse (Malenka et al., 1989; Malinow et al., 1989; Otmakhov et al., 1997; Chen et al., 2001). This ability to turn on and off the autophosphorylation of CaMKII distinguishes our experiments from some remarkable progress done with CaMKII mutants. Mice with a point mutation of the threonine at the position 286 of the αCaMKII gene show no NMDA-LTP and impaired spatial memory performance (Silva et al., 1992; Giese et al., 1998; Elgersma et al., 2004). However, to answer our question, the inactivation of CaMKII autophosphorylation was temporally restricted so it could have an effect during the stimulation of a single population of synapses. Only in this way we could observe the effect of KN93 in this form of heterosynaptic plasticity. The effects of KN-93 on other CaM Kinases are analyzed and discussed in section 6.3.2 and Supplemental data.

6.3 Results

The Input Output curves and the selection of the test stimulation frequencies were done as described in chapter 2 and 3 (section 3.3.1). Control traces are identical to those depicted in the Chapter 2 Fig. 2.6

6.3.1 KN93 at 10 μM blocks the late phase of LTP

10 μM KN-93 blocked L-LTP at 10 h if present at the time of induction (pathway S2 in Fig. 6.3), but had no effect on the induction of LTP on a pathway tetanized 20 min earlier (pathway S1 in Fig 6.3). In separate experiments, the order was reversed and the same dose of KN-93 was present during the first tetanization but then washed out. L-LTP on the pathway tetanized under KN-93 failed to show any potentiation 10 h post-tetanus when compared to a control baseline (pathway S1 in Fig. 6.5). However, after 20 min of aCSF flow into the chamber that successfully washed out KN-93, tetanization of the second pathway induced robust L-LTP lasting 10 h (pathway S2 in Fig. 6.5).

Figure 6.2 Representative fEPSPs waveforms from an individual 'Strong before Strong with 10μM KN-93' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.3A

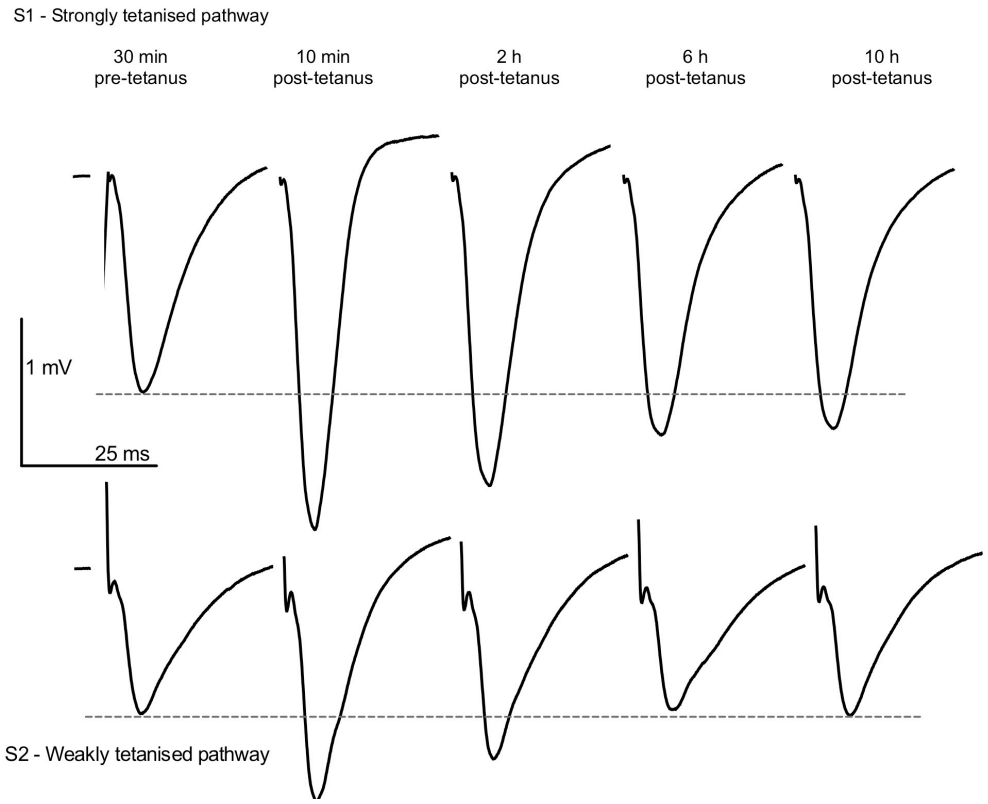


Figure 6.3 10 μM KN-93 blockage of L-LTP in S2 is not prevented by PRPs available to S1.

A. An individual experiment showing a representative example of the blockade of L-LTP by 10 μM KN-93 (S2) despite the L-LTP obtained by the strong tetanization of another pathway (S1).

B. Grouped data for experiments in which S2 received strong tetanisation and the resulting potentiation was maintained for less than 10 h when compared to the control pathway. 10 μM KN-93 blocks L-LTP if present at the time of induction (pathway S2 compared to baseline at 10 h ($t = 0.4$, $p > 0.05$)), but not during the maintenance of already induced LTP (pathway S1 ($t = 3.9$, $p < 0.01$)) ($n = 7$).

C. Statistical comparisons were made between the strongly-tetanised pathway (S2) under the presence of 10 μM KN-93 and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope, expressed as the normalised percentage change, and measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 11.29$, $p < 0.001$), after 2 h ($t = 4.95$, $p < 0.001$) but not after 6 h ($t = 0.54$, $p > 0.05$) or after 10 h ($t = 0.42$, $p > 0.05$).**

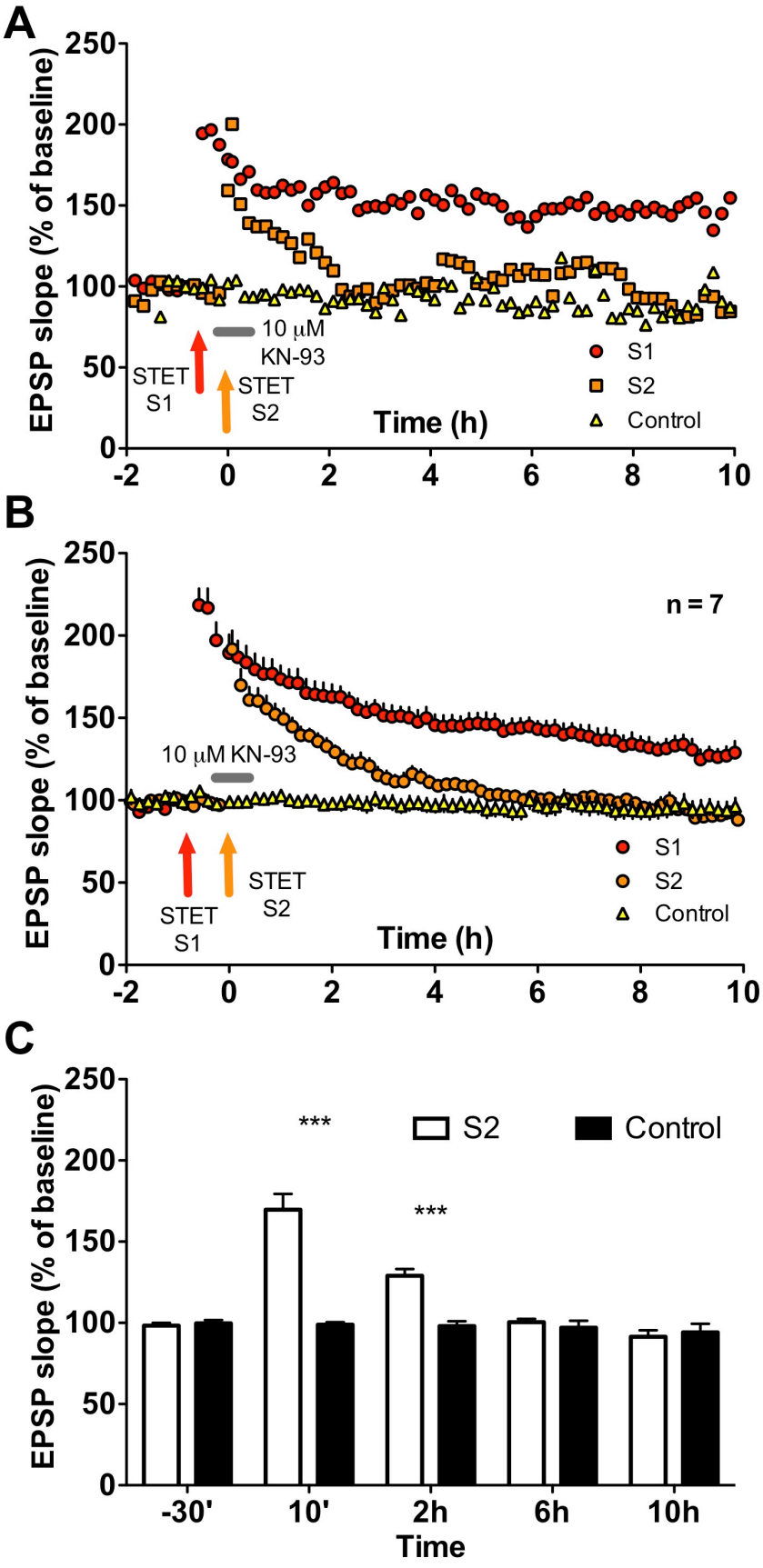

A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
10 μM
KN-93
STET
S1
STET
S2
Time (h)
S1
S2
Control
B
EPSP slope (% of baseline)
n = 7
10 μM KN-93
STET
S1
STET
S2
Time (h)
S1
S2
Control
C
EPSP slope (% of baseline)
***
***
S2
Control
-30'
10'
2h
6h
10h
Time

Figure 6.4 Representative fEPSPs waveforms from an individual 'Strong with 10 μM KN-93 before Strong experiment'.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.5

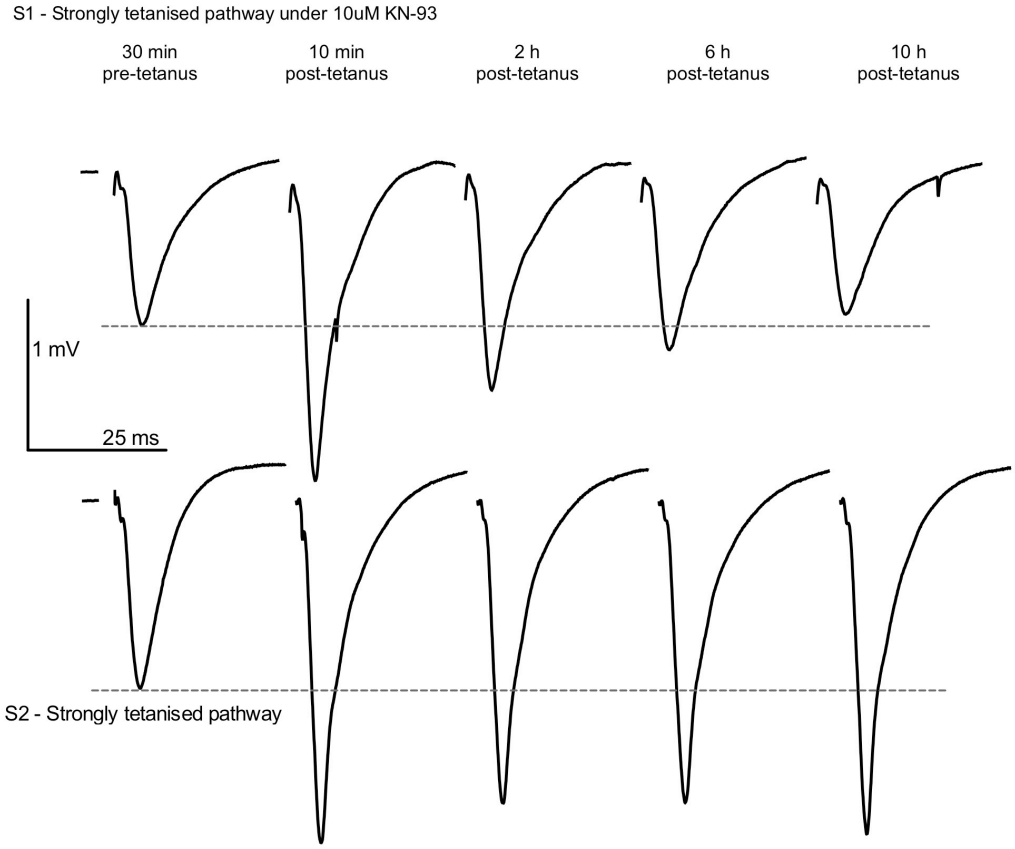
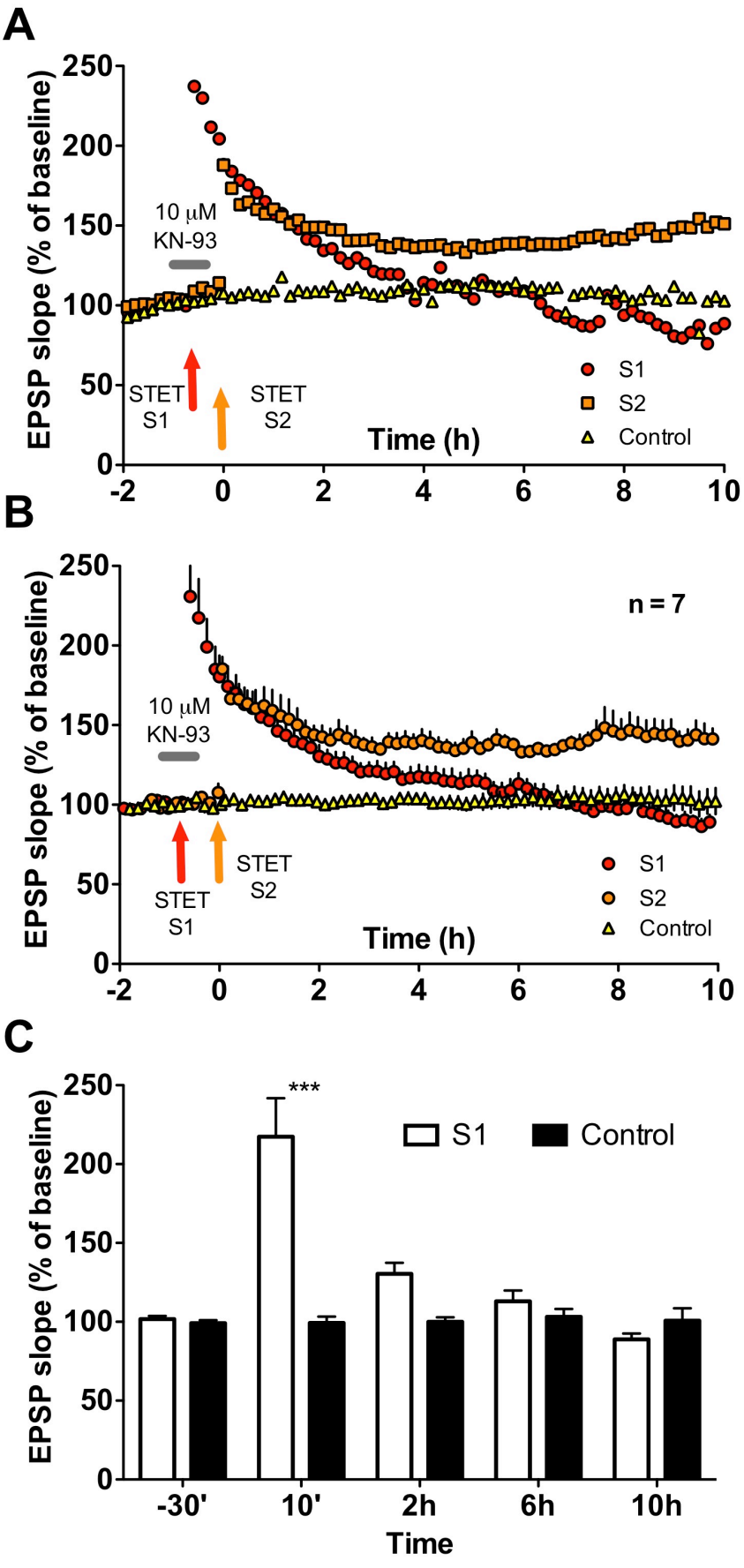


Figure 6.5 10 μM KN-93 block of L-LTP in S1 is not prevented by PRPs available to S2.

A. An individual experiment showing a representative example of the blockade of L-LTP by 10 μM KN-93 (S1) despite the L-LTP obtained by the strong tetanization of another pathway (S2).

B. Same as in Fig 6.3 but the pathway under the influence of KN-93 was stimulated first (pathway S1 vs. control pathway at 10 h ($t = 1.2$, $p > 0.05$)), the drug then washed out and stimulation given to another independent pathway which successfully expressed L-LTP (pathway S2 vs. control pathway ($t = 3.4$, $p < 0.01$) ($n = 7$).

C. Statistical comparisons were made between the strongly-tetanised pathway (S1) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed after 10 min ($t = 9.18$, $p < 0.001$), but not after 2 h ($t = 2.36$, $p > 0.05$) or after 6 h ($t = 0.77$, $p > 0.05$) or after 10 h ($t = 0.92$, $p > 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
10 μM
KN-93
STET
S1
STET
S2
Time (h)
S1
S2
Control
-2
0
2
4
6
8
10
B
n = 7
10 μM
KN-93
STET
S1
STET
S2
Time (h)
S1
S2
Control
C
***
S1
Control
-30'
10'
2h
6h
10h
Time

6.3.2 KN93 at 1 μM blocks L-LTP, but allows a separate and weakly tetanized pathway to display L-LTP

The results reported in the previous section support a role of CaMKII in the setting of the tag. The question remains, however, as to whether the inhibition of CaMKII with 10 μM KN-93 also blocks the pathways responsible for the availability of PRPs. 'Strong before Strong' experiments are unable to discriminate between a block of the tag and a general inhibition of both the tag and the PRPs (section 1.4.5, Fig. 1.3) To discriminate tag setting from PRP synthesis or availability, a low concentration of KN-93 (1 μM) was applied to a strongly tetanized pathway (S1, to block L-LTP), washed out and 20 min later, a weak tetanization protocol given to pathway S2 ('tag-block' protocol (Fig. 1.4)). This concentration of KN-93 has previously been shown to be sufficient to impair synaptic plasticity in brain-slices (Hansel et al., 2006) and CaMKII, with a Ki of 370 nM (Sumi et al., 1991), should be effectively blocked, while the activity of other CaM kinases, such as CaMKIV, less potently inhibited (Ishida et al., 1995). The lower dose of KN-93 was chosen in order to not interfere with the CaMKK pathway which was expected to be necessary for the synthesis of PRPs (see chapter 7). To support our choice of 1 μM KN-93, the effect of different concentrations of KN-93 on CaMKII and CREB phosphorylation was assessed in hippocampal dissociated cultures by our collaborators Hiroyuki Okuno and Haruhiko Bito. The results have been included in this thesis as supplemental data (Figure S1). They support a concentration-dependent inhibitory effect of KN-93 on the autophosphorylation of CaMKII and the activation of CREB. Importantly, a higher concentration of KN-93 is required to inhibit CREB activation than to inhibit CaMKII. What would be the effect of a low dose of KN-93 on a 'tag-block experiment' (Fig. 1.4)?

In the experiments descrbied in this section, a low concentration (1 μM) of KN-93 completely blocked L-LTP on pathway S1 (Fig. 6.7). However, pathway S2 weakly stimulated 20 min after KN-93 washout maintained L-LTP for at least 10 h (Fig. 6.7) and was stable from 2 to 10 hours. This is a critical observation for it implies that PRPs must have been available to synapses of the S2 pathway. As S2 was weakly tetanized, these could only have been upregulated by the strong tetanization of

pathway S1. Given that pathway S1 decayed to baseline, it follows that 1 μM KN-93 selectively blocks tag setting but not PRP availability.

In a 'weak-before-strong' protocol (a mirror version of the 'tag-blobk' protocol (Fig. 1.4)), the weakly tetanized pathway (S2) showed sustained L-LTP over 10 h, despite the decay to baseline (and cross-over) of the potentiation induced by strong tetanization in the presence of KN-93 of the S1 pathway (Fig. 6.9). Thus, at 1 μM, KN-93 blocks tag setting but allows PRPs to be synthesized and made available. Together, these experiments suggest that CaMKII, being sensitive to lower concentrations of KN-93, might play a major role in tag setting whereas its role in the regulation of PRP availability might be more limited.

Figure 6.6 Representative fEPSPs waveforms from an individual 'Strong with 1µM KN-93 before Weak' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms).

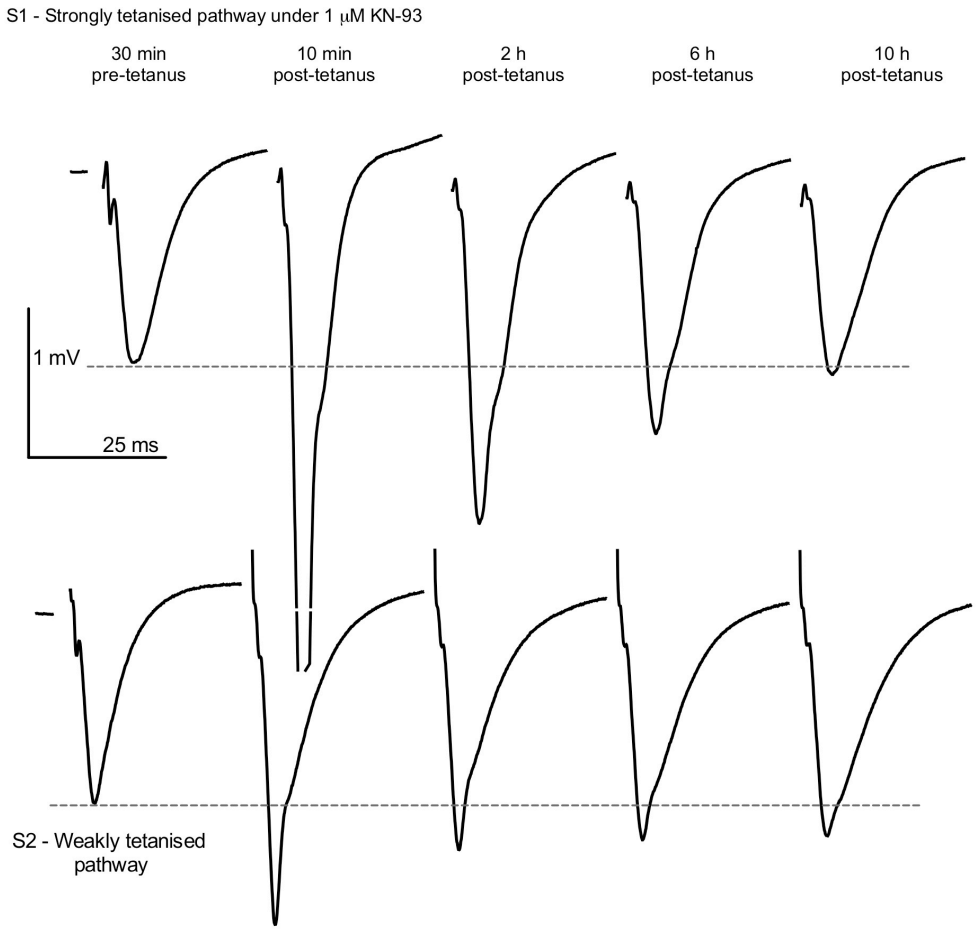


Figure 6.7 1 μM KN-93 blocks L-LTP in S1 but PRPs are available to S2.

A. An individual experiment showing a representative example of the rescue of E-LTP into L-LTP (S2) by prior heterosynaptic strong-tetanisation under the influence of 1 μM KN-93 (S1).

B. Grouped data for experiments in which strong tetanization in the presence of 1 μM KN-93 (pathway S1) induces LTP that decays to baseline over 10 h (S1 vs. control pathway; $t = 1.7$, $p > 0.05$; red symbols), while an independent set of weakly tetanized synapses (S2; orange symbols) successfully shows stable potentiation for 10 h post-tetanus (S2 relative to baseline at 10 h post-tetanus; ($t = 3$, $p < 0.05$) and S2 2 h to 10 h ($t = 1.2$, $p > 0.05$)) ($n = 8$).

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 5.58$, $p < 0.001$), and after 2 h ($t = 2.845$, $p < 0.05$) but not after 6 h ($t = 2.286$, $p > 0.05$) or after 10 h ($t = 2.615$, $p > 0.05$).**

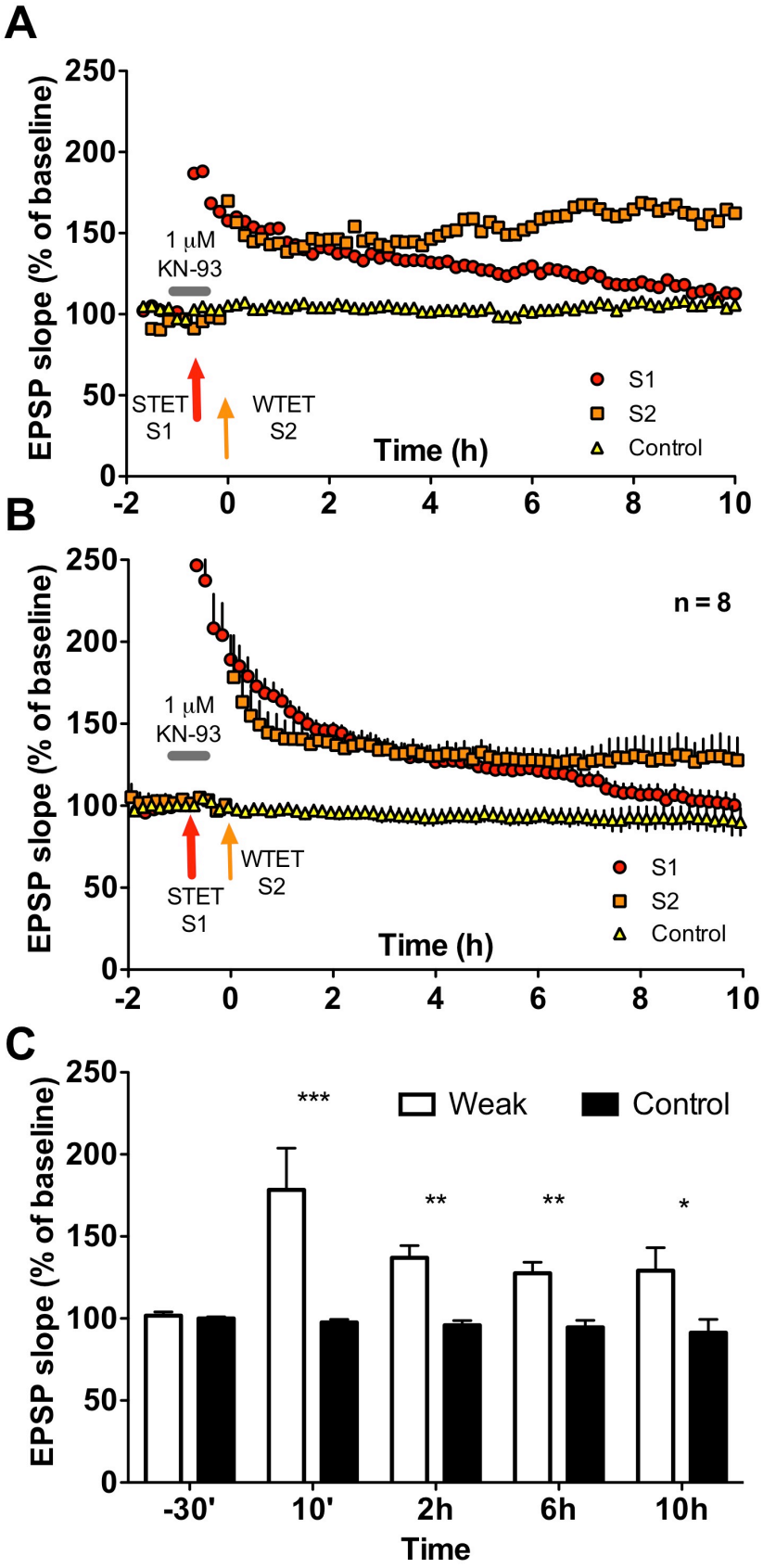

A
EPSP slope (% of baseline)
250
200
150
100
50
0
1 μM
KN-93
STET
S1
WTET
S2
Time (h)
S1
S2
Control
-2
0
2
4
6
8
10
B
n = 8
C
***
**
**
*
Weak
Control
-30'
10'
2h
6h
10h
Time

Figure 6.8 Representative fEPSPs waveforms from an individual 'Weak before Strong with 1 µM KN-93 experiment'.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.9

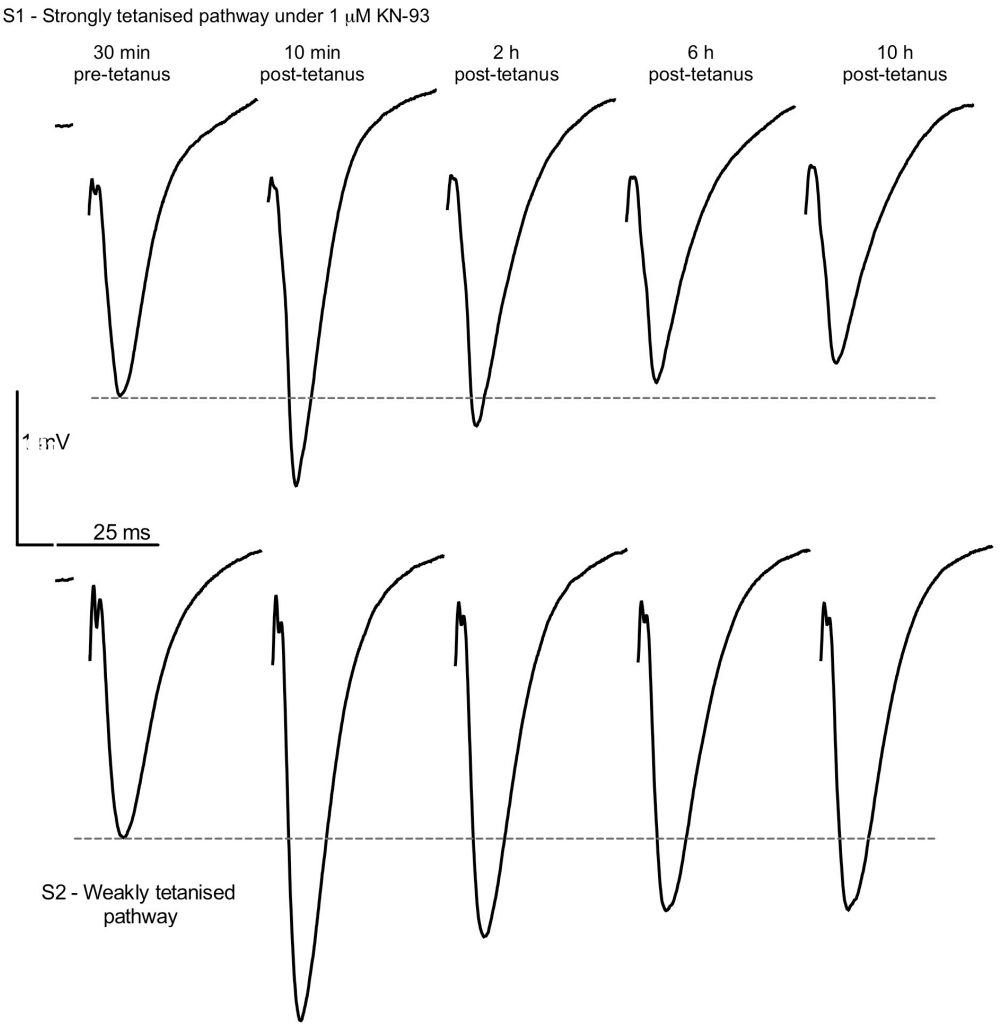
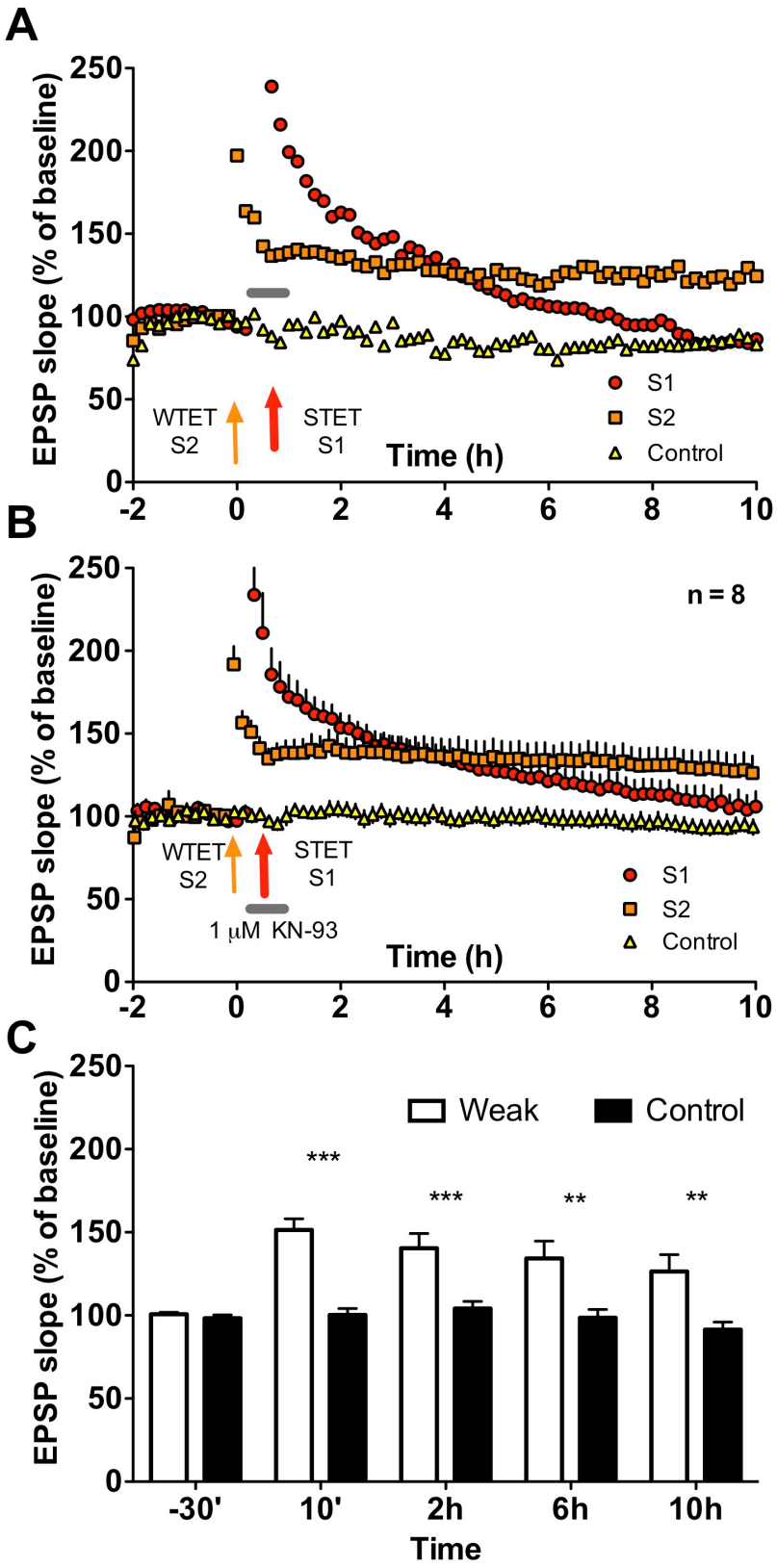


Figure 6.9 1 μM KN-93 blocks L-LTP in S2 but PRPs are available to S1.

A. An individual experiment showing a representative example of the rescue of E-LTP into L-LTP (S2) by subsequent heterosynaptic strong-tetanisation under the influence of 1 μM KN-93 (S1).

B. Grouped data for experiments in which in a 'weak-before-strong' protocol, early-LTP is still rescued to L-LTP (S2 vs. control pathway; $t = 3.2$, $p < 0.05$) even though L-LTP fails to be maintained in those synapses tetanized in the presence of 1 μM KN-93 (S1 vs. control pathway; $t = 1.4$, $p > 0.05$; S1 2 h vs. 10 h; $t = 4.7$, $p < 0.01$)) ($n = 7$). Error bars indicate SEM. Symbols as in Fig 3.1.

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 5.59$, $p < 0.001$), after 2 h ($t = 3.97$, $p > 0.001$) after 6 h ($t = 3.92$, $p < 0.01$) and after 10 h ($t = 3.82$, $p < 0.01$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
WTET
S2
STET
S1
Time (h)
S1
S2
Control
B
n = 8
1 μM KN-93
C
Weak
Control
***
***
**
**
-30'
10'
2h
6h
10h
Time

6.3.3 KN93 at 10 μM blocks L-LTP and does not allow a separate and weakly tetanized pathway to display L-LTP

Based on the biochemical data gathered in hippocampal cell cultures and presented as supplemental data (Fig. S1), we predicted that at a higher concentration (10 μM), KN-93 would not affect tag-setting selectively as occurred at 1 μM. In the 'strong-before-weak' protocol (i.e. 'tag-block' Fig. 1.4), KN-93 still blocked L-LTP of the strongly stimulated pathway S1 but now, pathway S2 that was weakly tetanized in the absence of KN-93 also failed to maintain its potentiation (Fig. 6.11). Similarly, using the 'weak-before-strong' protocol, KN-93 successfully blocked L-LTP in the strongly tetanized pathway while pathway S2 also failed to show L-LTP (pathway S2 Fig. 6.13).

Figure 6.10 Representative fEPSPs waveforms from an individual 'Strong with 10 μM KN-93 before Weak' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.11

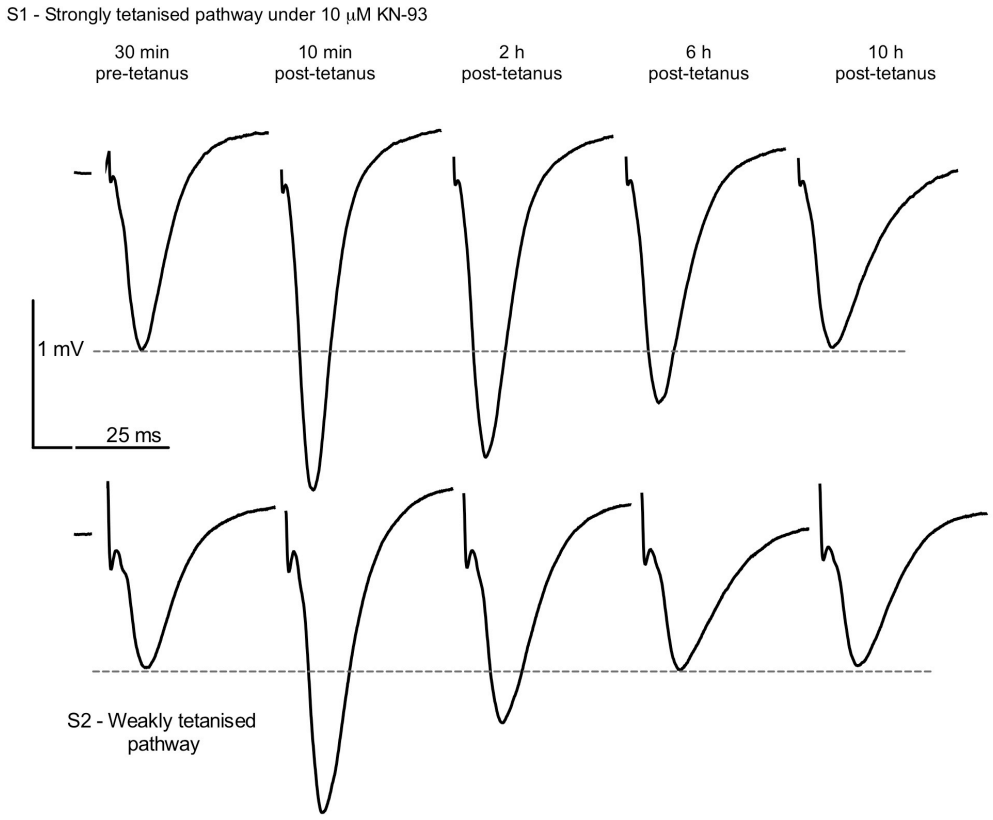


Figure 6.11 10 μM KN-93 blocks L-LTP in S1 and PRPs are not available to S2.

A. An individual experiment showing a representative example of the failure of heterosynaptic strong-tetanisation under the influence of 10 μM KN-93 (S1) to rescue E-LTP into L-LTP in a weakly-tetanised pathway (S2).

B. Grouped data for experiments in which in a weakly stimulated pathway (S2) failed to show L-LTP (S2 vs. control 10 h; $t = 0.1$, $p > 0.05$) was the stimulation was given 20 min after a strong tetanus to S1 if the higher concentration of 10 μM KN-93 was present during LTP induction. The strongly stimulated pathway also fails to show L-LTP (S1 vs. control 10 h; $t = 1.4$, $p > 0.05$)) ($n = 9$).

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 8.36$, $p < 0.001$), after 2 h ($t = 3.95$, $p > 0.001$) but not after 6 h ($t = 1.99$, $p > 0.05$) or after 10 h ($t = 0.03$, $p > 0.05$).**

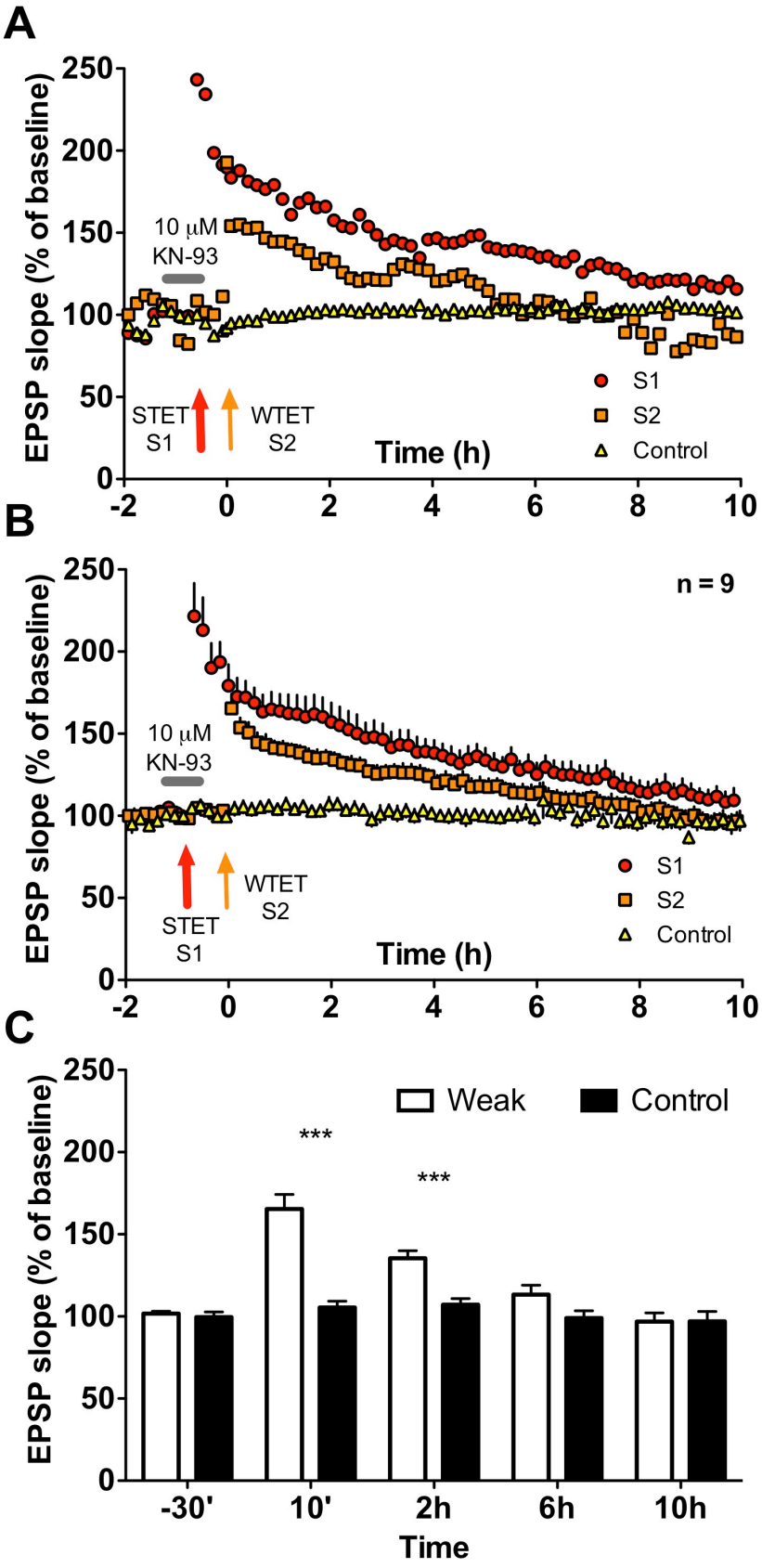

A
EPSP slope (% of baseline)
250
200
150
100
50
0
10 μM
KN-93
STET
S1
WTET
S2
Time (h)
S1
S2
Control
-2
0
2
4
6
8
10
B
n = 9
C
Weak
Control
***
***
-30'
10'
2h
6h
10h
Time

Figure 6.12 Representative fEPSPs waveforms from an individual 'Weak before Strong with 10 µM KN-93 experiment'.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms).

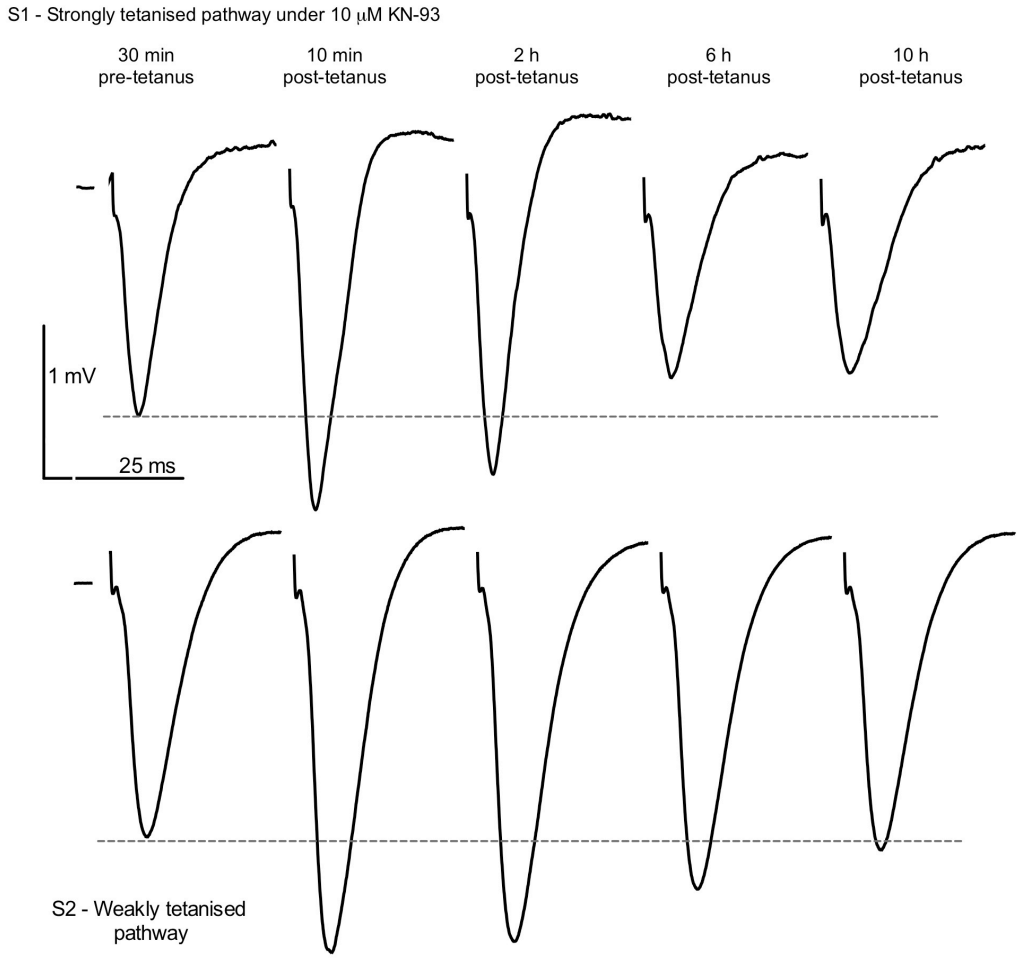
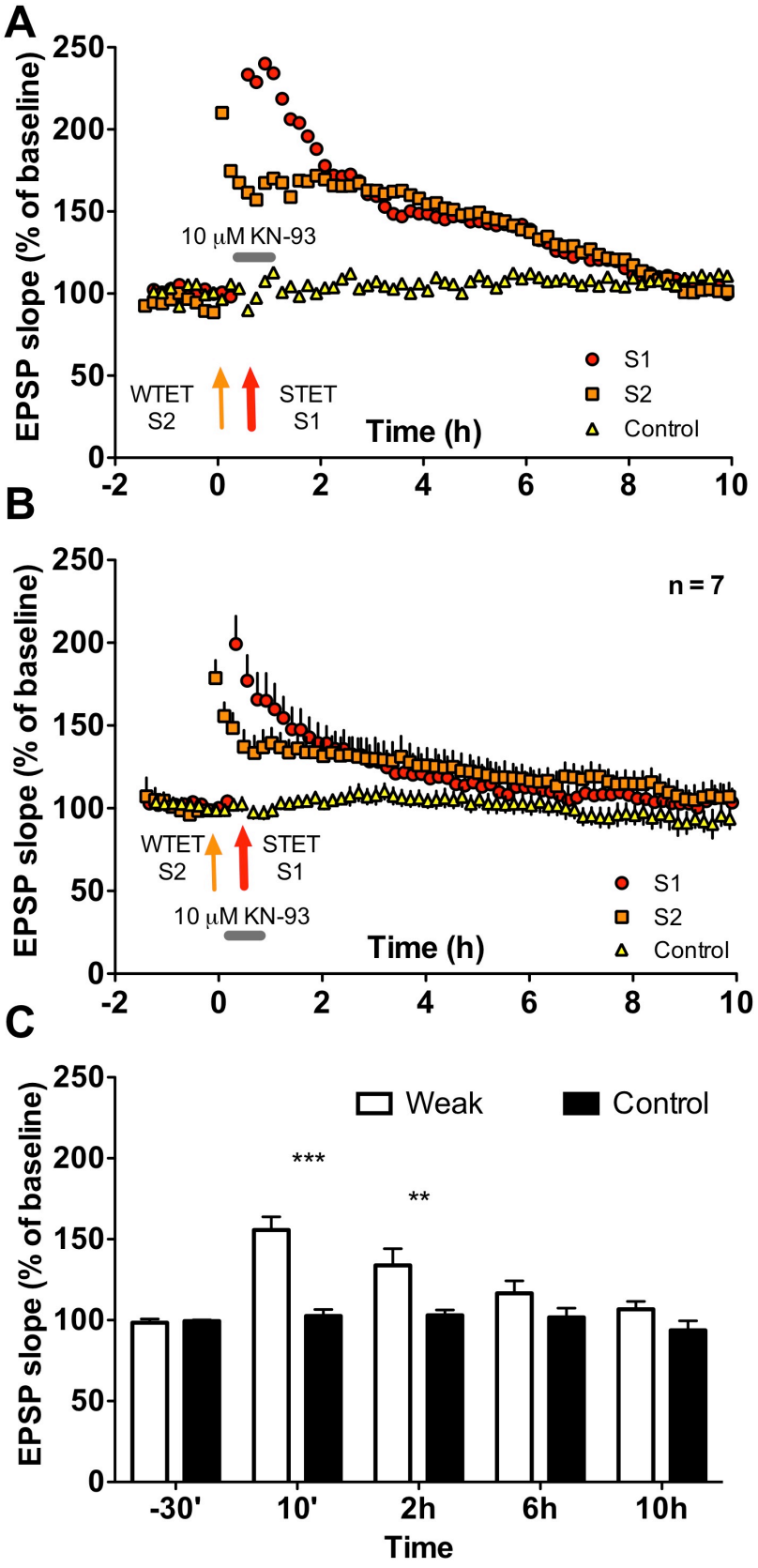


Figure 6.13 10 μM KN-93 blocks L-LTP in S2 and PRPs are not available to S1.

A. An individual experiment showing a representative example of the failure of heterosynaptic strong-tetanisation under the influence of 10 μM KN-93 (S1) to rescue E-LTP into L-LTP in a weakly-tetanised pathway (S2).

B. Grouped data for experiments in which in the rescue of early-LTP into L-LTP is also not seen in S2 (S2 vs. control 10 h; t test = 1.7, $p > 0.05$) if tetanization is given when 10 μM KN93 is present during S1. S1 also fails to maintain L-LTP (S1 vs. control 10h ($t = 0.9$, $p > 0.05$)) (n = 7).

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 6.28$, $p < 0.001$), after 2 h ($t = 3.65$, $p < 0.01$) but not after 6 h ($t = 1.75$, $p > 0.05$) or after 10 h ($t = 1.54$, $p > 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
10 μM KN-93
WTET
S2
STET
S1
Time (h)
S1
S2
Control
-2
0
2
4
6
8
10
B
n = 7
WTET
S2
STET
S1
10 μM KN-93
C
Weak
Control
***
**
-30'
10'
2h
6h
10h
Time

6.3.4 KN92 at 10 μM does not affect STC

To control for non-specific effects of KN-93, we tested the effect of the inactive analogue KN-92 (10 μM) on LTP and heterosynaptic plasticity. KN-92 did not impair L-LTP if present during strong tetanization (Fig. 6.15). Moreover, using a 'weak-before-strong' protocol, KN-92 did not prevent the rescue of early-LTP on a weakly tetanized pathway S2 when given in the presence of strong tetanization on S1 (Fig. 6.17).

Figure 6.14 Representative fEPSPs waveforms from an individual 'Strong with 10 µM KN-92 before Strong' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.15

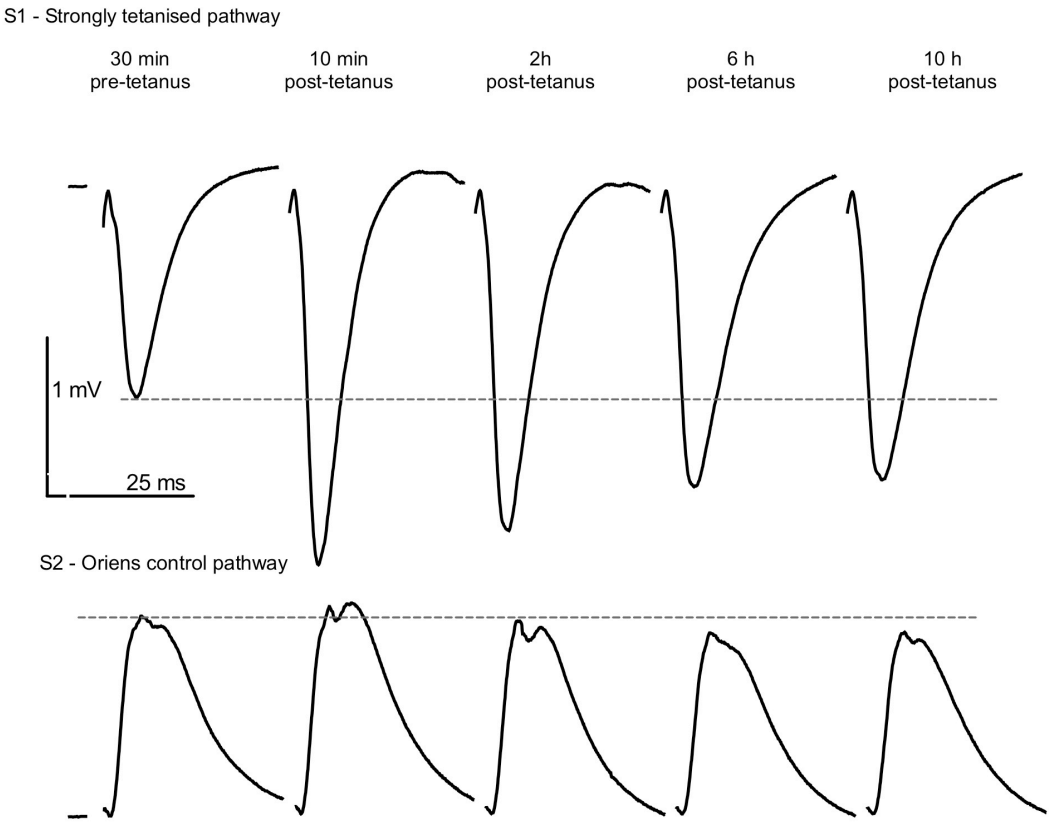


Figure 6.15 10 µM KN-92 does not impair L-LTP in S1.

A. An individual experiment showing a representative example of the success of heterosynaptic strong-tetanisation under the influence of 10 µM KN-92 to express and maintain L-LTP (S1).

B. Grouped data for experiments in which in a strong tetanus to S1 under the higher concentration of 10 µM KN-92 was present during LTP induction. Contrary to the effect of 10 µM KN-93 and 1 µM KN-93 shown previously, the strongly stimulated pathway succeeds in maintaining L-LTP (S1 vs. control 10 h; $t = 6.39$, $p < 0.001$)) ($n = 5$).

C. Statistical comparisons were made between the strongly tetanised pathway (S1) under the influence of KN-92 and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 11.37$, $p < 0.001$), after 2 h ($t = 7.17$, $p < 0.001$) after 6 h ($t = 6.32$, $p < 0.001$) and after 10 h ($t = 6.39$, $p < 0.001$).**

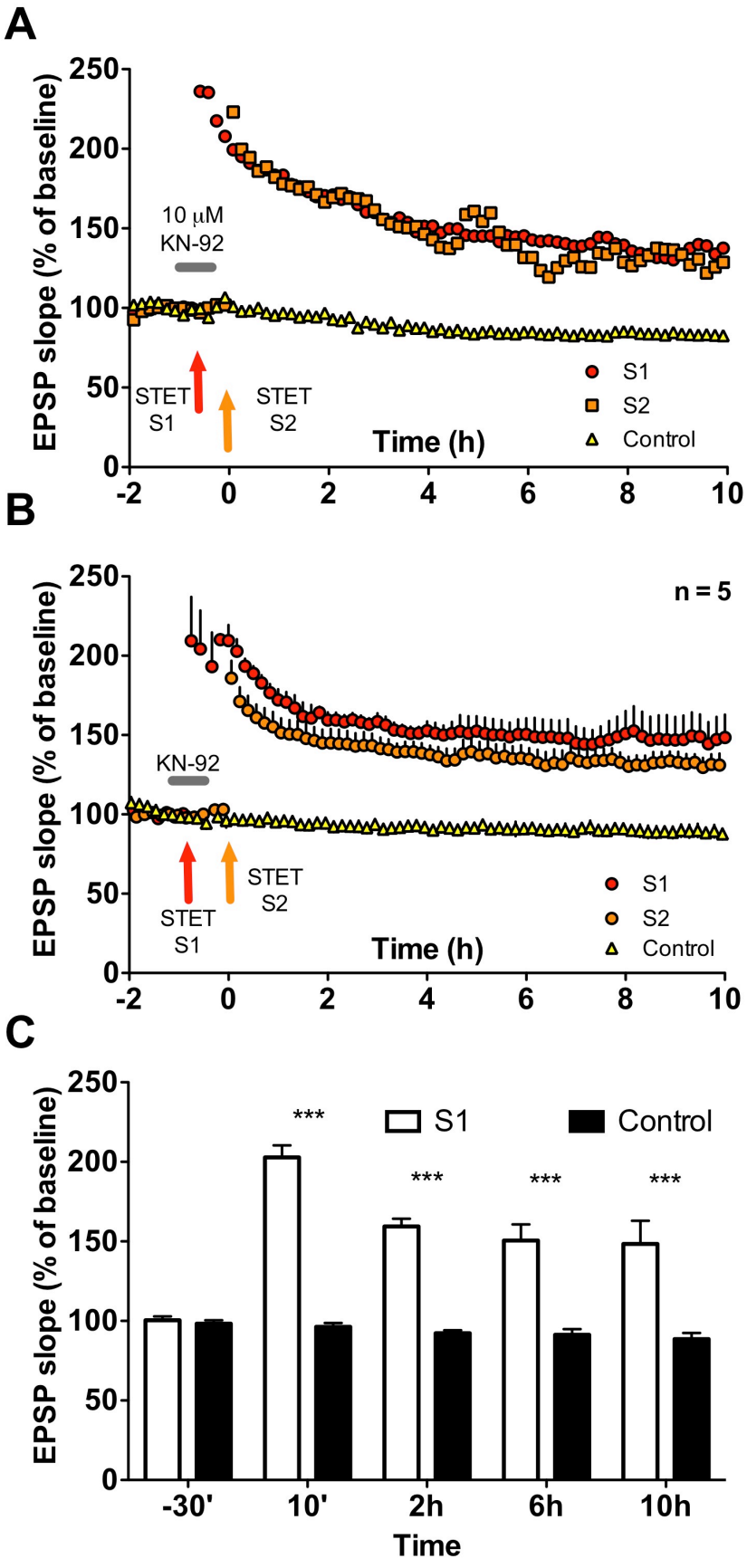

A
EPSP slope (% of baseline)
250
200
150
100
50
0
10 μM
KN-92
STET
S1
STET
S2
Time (h)
S1
S2
Control
-2
0
2
4
6
8
10
B
n = 5
KN-92
STET
S1
STET
S2
C
***
S1
Control
-30'
10'
2h
6h
10h
Time

Figure 6.16 Representative fEPSPs waveforms from an individual 'Weak before Strong with 10 μM KN-92 experiment'.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 6.13

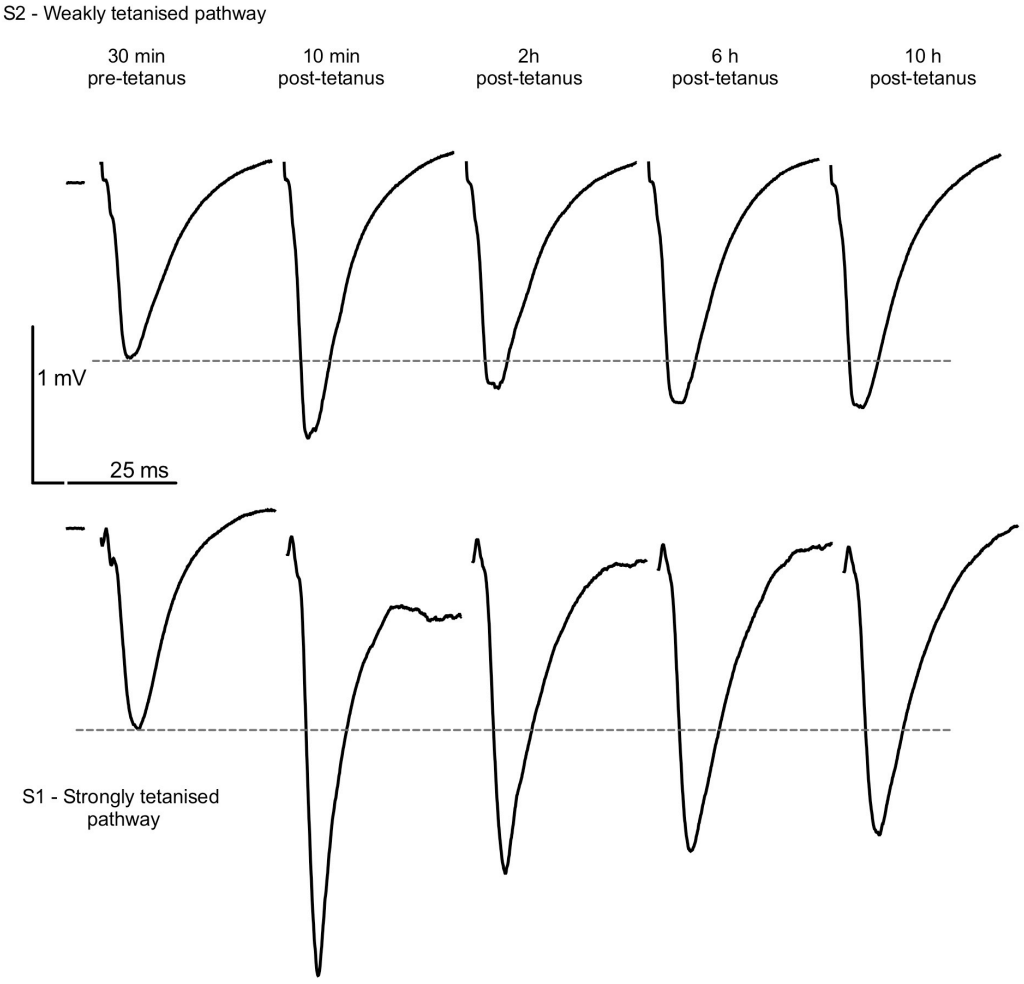
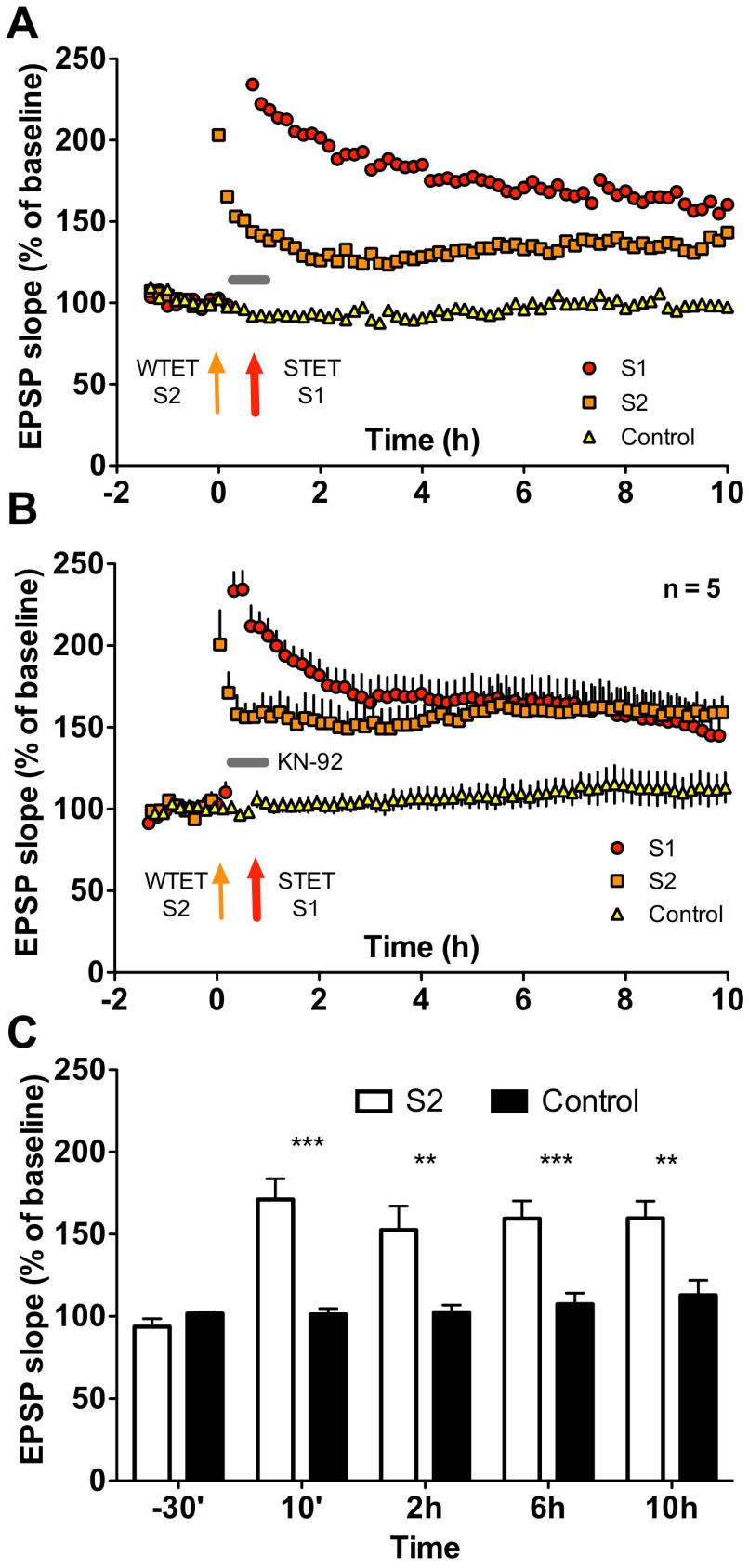


Figure 6.17 10 μM KN-92 does not block L-LTP (S1) and allows for heterosynaptic rescue of E-LTP into L-LTP (S2)

A. An individual experiment showing a representative example of the success of heterosynaptic strong-tetanisation under the influence of 10 μM KN-92 (S1) to rescue E-LTP into L-LTP in a weakly-tetanised pathway (S2).

B. Grouped data for experiments in which the rescue of early-LTP into L-LTP is also seen in S2 (S2 vs. control 10 h; t test = 3.77, $p < 0.01$) if tetanization is given when KN-92 (10 μM) is present during S1 (n = 5).

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 5.65$, $p < 0.001$), after 2 h ($t = 4.04$, $p < 0.01$), after 6 h ($t = 4.20$, $p < 0.001$) and after 10 h ($t = 3.77$, $p < 0.01$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
WTET
S2
STET
S1
Time (h)
S1
S2
Control
B
EPSP slope (% of baseline)
n = 5
KN-92
WTET
S2
STET
S1
Time (h)
S1
S2
Control
C
EPSP slope (% of baseline)
S2
Control
***
**
***
**
-30'
10'
2h
6h
10h
Time

6.4 Discussion

CaMKII is a broad range kinase that regulates many neuronal functions (Erondu and Kennedy, 1985; Braun and Schulman, 1995; Yamauchi, 2005). In the CA3 to CA1 Schaffer-collateral pathway of the hippocampus, CaMKII activation by Ca^{2+}/CaM is required at the time of LTP induction (Malenka et al., 1989), and, as shown recently, it remains necessary even during the maintenance of LTP (Sanhueza et al., 2007)). CaMKII may act as a switch capable of maintaining changes in synapse efficacy (Lisman and Goldring, 1988; Lisman and Zhabotinsky, 2001; Miller et al., 2005). This switch quality could, in principle, account for tag setting and/or the local capture of available PRPs.

This property of CaMKII is supported by our findings concerning the actions of KN-93. When strong tetanization was given to one pathway, an independent input subsequently and strongly stimulated under the influence of a high, 10 μM concentration of KN-93 failed to show L-LTP. That is, the second pathway fails to make use of the PRPs putatively supplied by the strong tetanization of the first pathway (Fig. 6.3). Sajikumar et al. (2007) before us (using KN-62), and now we, interpret this finding as potentially indicating the necessity of functional CaMKII activity for the process of tag setting (Sajikumar et al., 2007). If tags cannot be set at synapses that receive strong stimulation in the presence of KN-93, these synapses would be unable to capture the newly synthesized and available PRPs from the other pathway. The same logic applies to experiments in which 10 μM KN-93 is applied during the first strong tetanus (Fig. 6.5).

However, the weakness of the 'strong-before-strong' stimulation protocol is that it is not definitive with respect to all the molecular consequences of the CaMK inhibition. This is because, while KN-93 certainly blocks tagging, the drug may *also* prevent the synthesis or availability of PRPs - a second action that cannot be unmasked with the 'strong-before-strong' protocol (see chapter 1, section 1.4.5, Fig. 1.3). In contrast, our novel use of 'strong-before-weak' and 'weak-before-strong' protocols (i.e. 'tag-block protocols' Fig. 1.4), with KN-93 during strong tetanization, are definitive. For if the sole effect of the KN-93 was to limit tag setting, PRPs would still be synthesized and made available heterosynaptically. Thus, in these new

protocols the weakly tetanized pathway would - as it does - display L-LTP. KN93 at 1 μM still blocked L-LTP on the strongly tetanized pathway (Figs. 6.7 & 6.9). However, the potentiation produced on the weakly stimulated pathway persisted for 10 h, both in the 'strong-before-weak' and 'weak-before-strong' paradigms. In fact, the potentiation shown on the two pathways crossed over such that the strong potentiation declined to baseline while the weak remained stable, bearing out our use of separate measurements of strength and persistence (stability between 2 and 10 h described in figure legends and explained in chapter 2 section 2.5).

In addition, the decline to baseline of the strongly tetanized pathways over 6 to 10 h also indicates that early-LTP occurs even in the absence of a functional tag. This observation is consistent with a further dissociation between the mechanism of tag setting and that responsible for the induction and expression of early-LTP (Bortolotto and Collingridge, 1998). To summarize, our results support Hypothesis 1 (Fig. 6.1) and reject Hypothesis 2. KN-93 at 1 μM blocks a synapse specific process needed for L-LTP, but not early-LTP itself, while leaving a cell-wide mechanism of synthesis and trafficking of new proteins fully functional. Our cell-biological data showing differential KN-93 dose-response functions of pCaMKII and pCREB (Supplemental Fig. S1) is consistent with this interpretation. To further test the dose-dependent actions of KN-93, the 'tag-block' protocols were run using 10 μM KN-93 (Figs. 6.11 & 6.13). In this case, both the strongly tetanized synapses under the presence of KN-93 *and* the weakly tetanized pathway fail to maintain their potentiation. This result can be explained by the possible absence of PRPs that in control experiments (KN-92 Figs. 6.15 & 6.17) succeed in stabilizing the expression of synpaptic potentiation.

These results opened a further question: If KN-93 at a higher concentration disrupts the synthesis of PRPs through its actions on the CaMKK-CaMKIV pathway, could the synthesis of PRPs be specifically blocked (without affecting the tag setting) by interfering with the CaMKK pathway (Chapter 7)?

Chapter 7: Role of the calcium-calmodulin kinase IV pathway in protein synthesis-dependent long-term potentiation.

7.1 Introduction

In the cell soma, calcium entry triggers CaMKK that activates CaMKIV (Bito et al., 1996; Tokumitsu et al., 2002), a nuclear kinase capable of phosphorylating Ca^{2+}/cyclic AMP-response element binding protein (CREB) (Bito et al., 1996; Ho et al., 2000) and initiating the transcription of genes that synthesise the PRPs necessary for stabilizing LTP (Kang et al., 2001). Another way that CaMKK has been show to modulate gene transcription is through its actions on the ERK pathway, consequently reducing the activation of translation factors (Schmitt et al., 2005). Importantly, the block of ERK with STO609 was not obtained when CaMKII inhibitors were used. This offered a clue as to the potential dissociation between the roles of CaMKII and CaMKIV in synaptic plasticity and suggested the following experiments.

Other studies point to a synaptic role of CaMKK in LTP through its activation of CaMKI and the subsequent incorporation of calcium-permeable AMPARs onto the post-synaptic membrane (Guire et al., 2008). Here too, STO-609 is shown to have a small effect in the early phases of E-LTP, while allowing a nearly normal expression of LTP in the early-phases of LTP (< 40 min). Is this synaptic effect of STO-609 capable of blocking the setting of synaptic tags?

The experiments described in this chapter were aimed at testing whether the CaMKK pathway has a necessary role in LTP specific to the synthesis of PRPs (Fig. 7.1).

7.1.1 CaMKK and the pathway leading to the synthesis of PRPs

In this chapter, 3 pathway protocols were used to examine the contribution to L-LTP of neuronal CaM kinases distinct from CaMKII, namely the CaMKK-CaMKI/IV pathway, using the CaMKK inhibitor STO-609 (Tokumitsu et al., 2002). Hippocampal dissociated cultures confirm that STO-609 has little effect on CaMKII activity (Tokumitsu et al., 2002) while inhibiting CREB phosphorylation. Supplemental figure S2 presents data supporting the use of 5 μM STO-609 as an

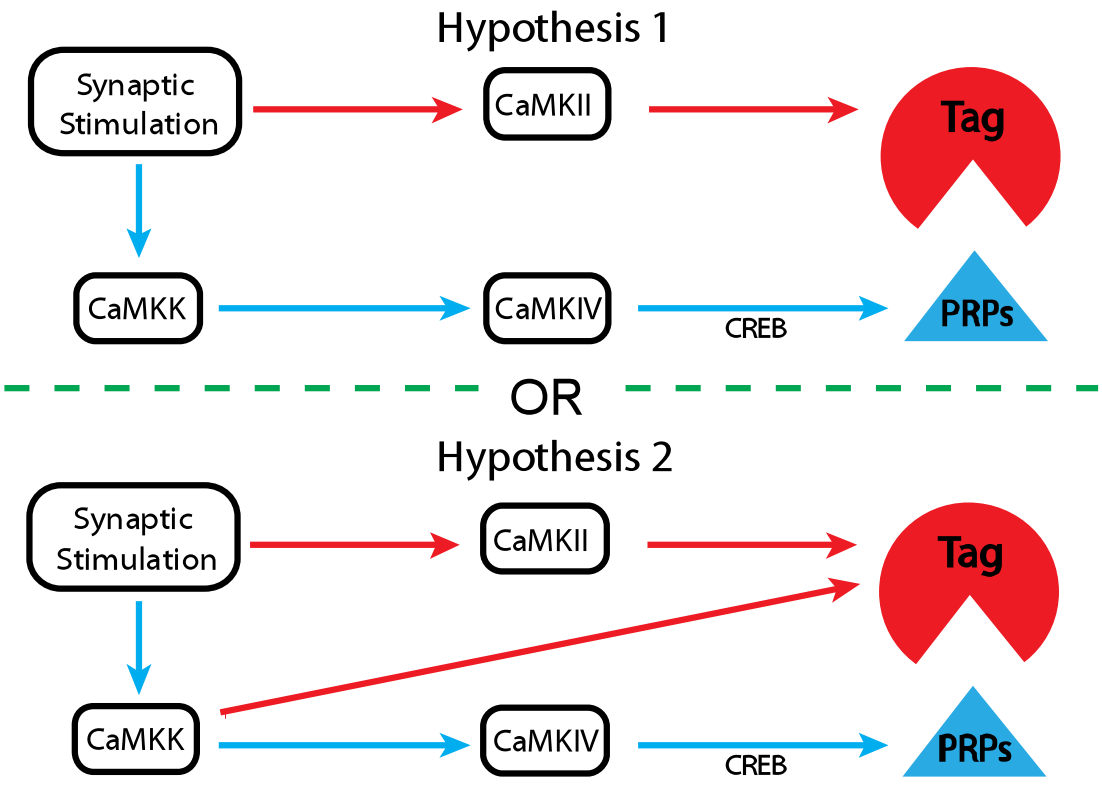
inhibitor of CREB phosphorylation while allowing CaMKII to autophosphorylate (Fig. S2). Does STO-609 block L-LTP through a specific effect on the availability of PRPs?

The hypothesis that CaMKK would have a necessary role for the synthesis of PRPs but not for the setting of local tags (Fig. 7.1) was challenged with the combination of experiments where STO-609, a CaMKK inhibitor, was used to block the maintenance of LTP. After confirming that STO-609 does indeed impair the late phases of LTP, the critical test involved the use of a 'weak before strong' paradigm where STO-609 was present during the weak tetanus but not during the delivery of another, heterosynaptic, strong tetanus. This 'weak before strong' paradigm is an alternative version of the 'test for PRP-block' described in chapter 1 (Fig. 1.3). The potential outcomes of this type of experiment would be informative as to whether STO-609 would have a role in the setting of a local tag in addition to the drug's alleged role in the block of PRP availability. In a 'weak before strong' protocol, the strong tetanus is capable of triggering the synthesis of PRPs that later are distributed to both strong and weakly stimulated synapses (Chapter 5). In this experiment, if the weakly stimulated synapses, under the presence of STO-609, failed to capture the PRPs made available by the strong, therefore failing to stabilize the E-LTP into L-LTP, one could claim an additional role to that of synthesis of PRPs to STO-609, and CaMKK. Under the framework of the STC hypothesis, that role would have to be necessary for the setting of the tag. As we shall see in this chapter, STO-609 does block L-LTP, but fails to block the rescue of E-LTP into L-LTP in the 'weak before strong' protocol. This supports the view that CaMKK, inhibited by STO-609, is a molecule with a necessary role for the availability of PRPs but with a null or redundant role in the setting of synaptic tags.

A series of controls are necessary to sustain the aforementioned conclusion. First, we confirmed that STO-609 had no effect on the progress of E-LTP induced by weak tetanisation (Fig. 7.7). Additionally, the successful wash-out of STO-609 was tested by delivering heterosynaptic strong stimulation with the same delay as in the 'weak before strong' experiment after a strong tetanus under the influence of STO-609. In this case, both sets of synapses succeed in maintaining L-LTP (Fig. 7.9).

Figure 7.1 Outline of the hypothesis.

Is the necessary role of CaMKK in LTP limited the signalling pathway leading to the synthesis of PRPs (Hypothesis 1) or is CaMKK activity also necessary on the signalling pathway leading to the setting of the tag?



7.2 Methods

Similar procedures for the preparation and incubation of slices were used as described in Chapters 2 and 3.

7.2.1 Preparation of slices, and recording set-up

Artificial cerebrospinal fluid (aCSF) was prepared with the following concentrations: NaCl 124 mM, KCl 3.7 mM, KH_2PO_4 1.2 mM, $MgSO_4(7H_20)$ 1.0 mM, $CaCl_2$ 2.5 mM, $NaHCO_3$ 24.6 mM, D-glucose 10 mM (pH = 7.4). 7 to 8 week old male Wistar rat was anaesthetized with halothane or isofluorane, before the brain was removed and sectioned as described previously (Leutgeb et al., 2003). 400 μm thick brain slices were sectioned with a Vibratome (Campden Instruments Integraslice 7550 PSDS) using stainless steel blades (Campdem Instruments 7550/1/SS). The brain slices were kept in a resting chamber with oxygenated aCSF for less than 5 min before being transferred into the experimental chamber. Three monopolar stainless steel stimulating electrodes (A-M systems) and the one stainless steel recording electrode were positioned as in Figure 2.1. The rate of stimulation provided 1 data point per stimulated channel every 2.5 min. For three pathway experiments this means that one channel is stimulated every 50 seconds (0.02 Hz).

7.2.2 Tetanus protocol

L-LTP was induced with the strong stimulation delivered using the protocol described in chapter 3 and consisted of 3 trains of 100 pulses at 100Hz delivered 10 min apart. E-LTP was elicited using the weak stimulation protocol described in chapter 4 and consisted of 20 pulses delivered in bursts of 5 pulses 100 Hz, 200 ms apart.

7.2.3 Drugs

STO-609 was purchased from Tocris and dissolved in aCSF with the help of less than 0.1% DMSO (Tokumitsu et al., 2002). 7H-benzimidazo[2,1-a]benz[de]isoquinoline-7-one-3-carboxylic acid (STO-609) is highly selective for

CaMKK of which it inhibits both its activity and its autophosphorylation. STO-609 has no effect on CaMKI and CaMKIV, and shows an IC50 about 100 times larger for CaMKII (90 μg/ml for CaMKKα vs. 10 μg/ml for CaMKII). It is also cell permeable which makes bath application possible.

7.3 Results

The Input Output curves and the selection of the test stimulation frequencies were done as described in chapter 2 and 3 (section 3.3.1). Control traces are identical to those depicted in the Chapter 2 Fig. 2.6

7.3.1 STO-609 at 5 μM blocks L-LTP.

The first step in the process to identify the mechanism of action of a drug in the STC framework is to confirm that it can block LTP. STO-605 was bath-applied to hippocampal slices and the 'strong tetanus' stimulation protocol was delivered. The resulting potentiation slowly decayed to baseline levels and was not stable when comparing the 2 h post-tetanus time point with the potentiation left after 10 h. (Fig. 7.3)

Figure 7.2 Representative fEPSPs waveforms from an individual 'Strong with STO-609' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 7.3

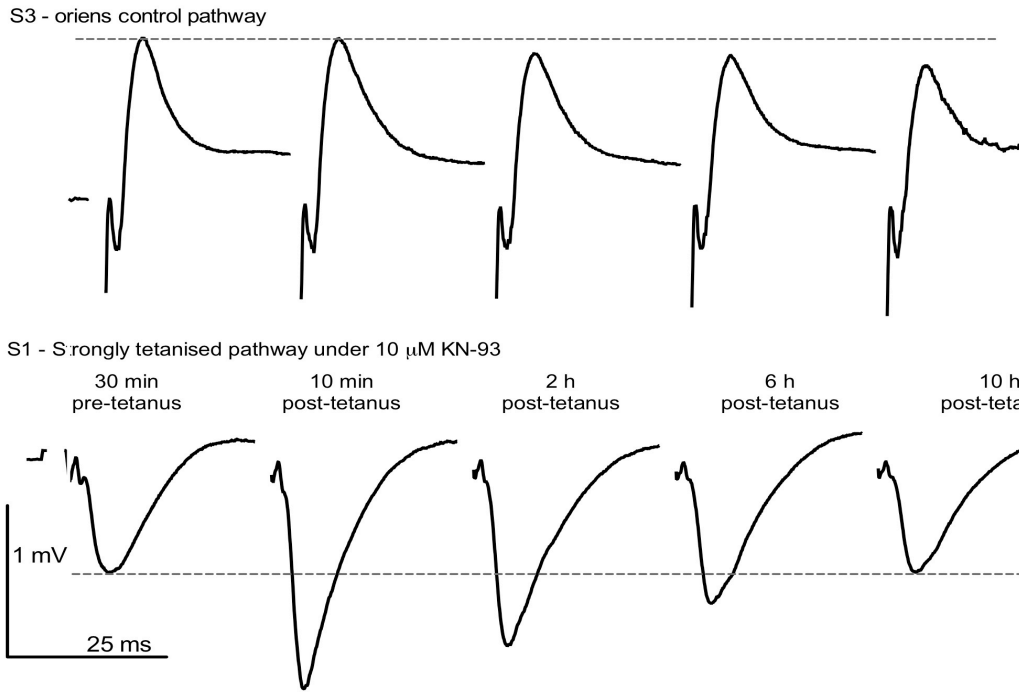
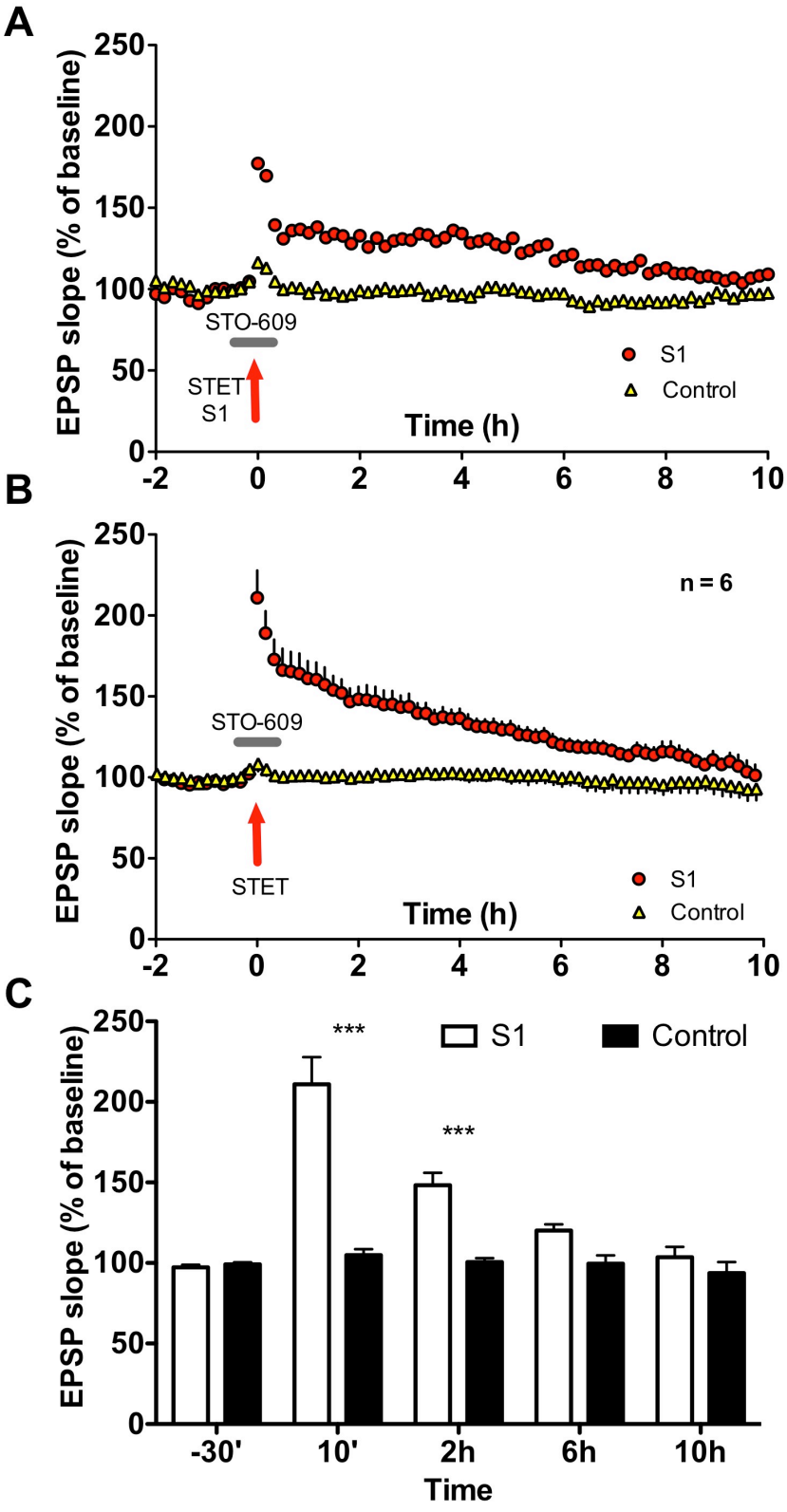


Figure 7.3 5 μM STO-609 block L-LTP.

A. An individual experiment showing a representative example of the failure of heterosynaptic strong-tetanisation under the influence of 5 μM STO-609 (S1) to show L-LTP.

B. Grouped data for experiments in which L-LTP is not maintained in S1 (S1 vs. control pathway 10 h; t = 1, p > 0.05) (n = 6). There is no stabilization of LTP after two hours (S1 2h vs. S1 10h; t = 8.04, p < 0.001).

C. Statistical comparisons were made between the strongly tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with p < 0.05; ** indicates p < 0.01; * indicates p < 0.001 (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed after 10 min (t = 10.58, p < 0.001), after 2 h (t = 4.75, p < 0.001) but not after 6 h (t = 2.05, p > 0.05) or after 10 h (t = 0.98, p > 0.05).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
STO-609
STET
S1
S1
Control
Time (h)
-2
0
2
4
6
8
10
B
EPSP slope (% of baseline)
n = 6
STO-609
STET
S1
Control
Time (h)
C
EPSP slope (% of baseline)
***
***
S1
Control
-30'
10'
2h
6h
10h
Time

7.3.2 E-LTP is rescued into L-LTP with subsequent strong heterosynaptic stimulation even if the weak tetanus is given under the presence of STO-609.

The STC hypothesis dissociates between mechanisms responsible for the setting of synaptic tags and those necessary for the synthesis of PRPs. The block of any of these two requirements can account for the decay of LTP. In the previous section STO-609 succeeds in blocking LTP. Which pathway is STO-609 blocking? To test the hypothesis that STO-609 is blocking exclusively the pathway leading to the synthesis of PRPs, the drug was tested in a 'PRP-block' protocol (Fig. 1.4). The particular version of the protocol used in the experiments described in this section consists on the induction of LTP with a 'weak tetanus' protocol under the influence of STO-609, followed by the washout of the drug and the subsequent induction of LTP with a 'strong tetanus'. The strongly tetanized synapses should have no problem expressing and maintaining their potentiated state since the strong tetanus would have engaged the synthesis of PRPs. The informative pathway is the 'weakly tetanized' pathway. Interstingly, this pathway succeeds in maintaing its potentiation suggesting that it was capable of making use of the PRPs available (Fig. 7.5). The effect of STO-609 seen in the previous section (Fig. 7.3) is rescued by the provision of PRPs, suggesting that STO-609 does not block LTP by interfering with the setting of local tags but by the disruption of the signalling leading to the availability of PRPs.

Figure 7.4 Representative fEPSPs waveforms from an individual 'Weak with STO-609 before Strong' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 7.5

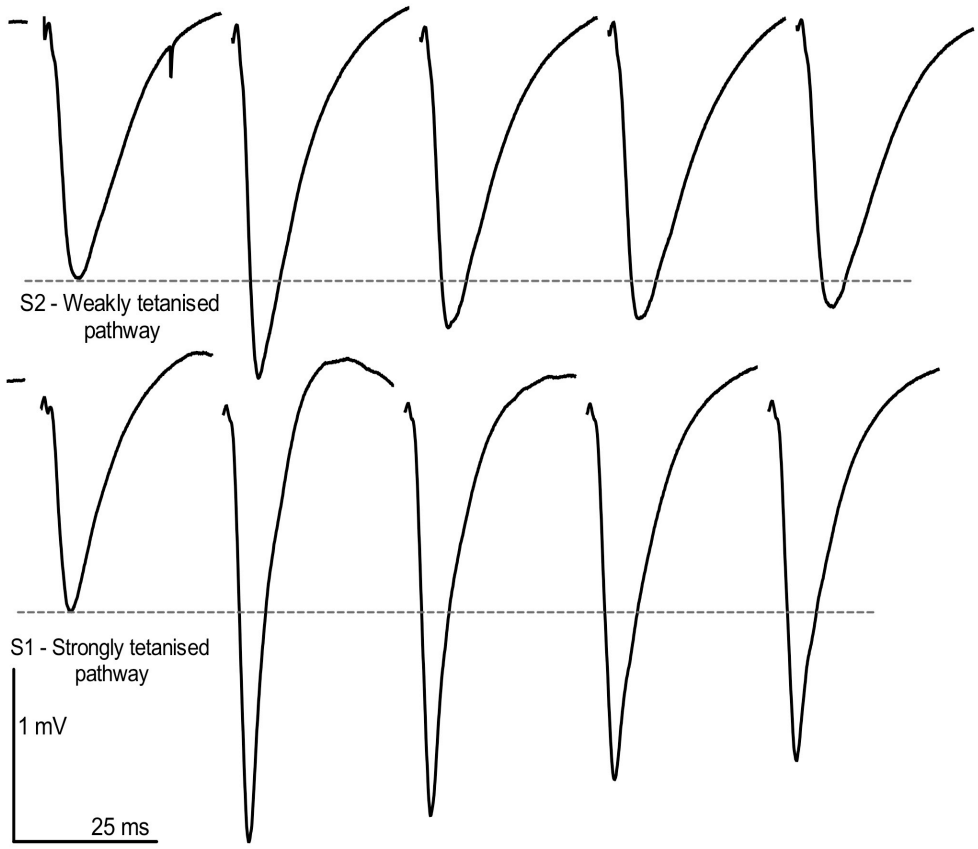
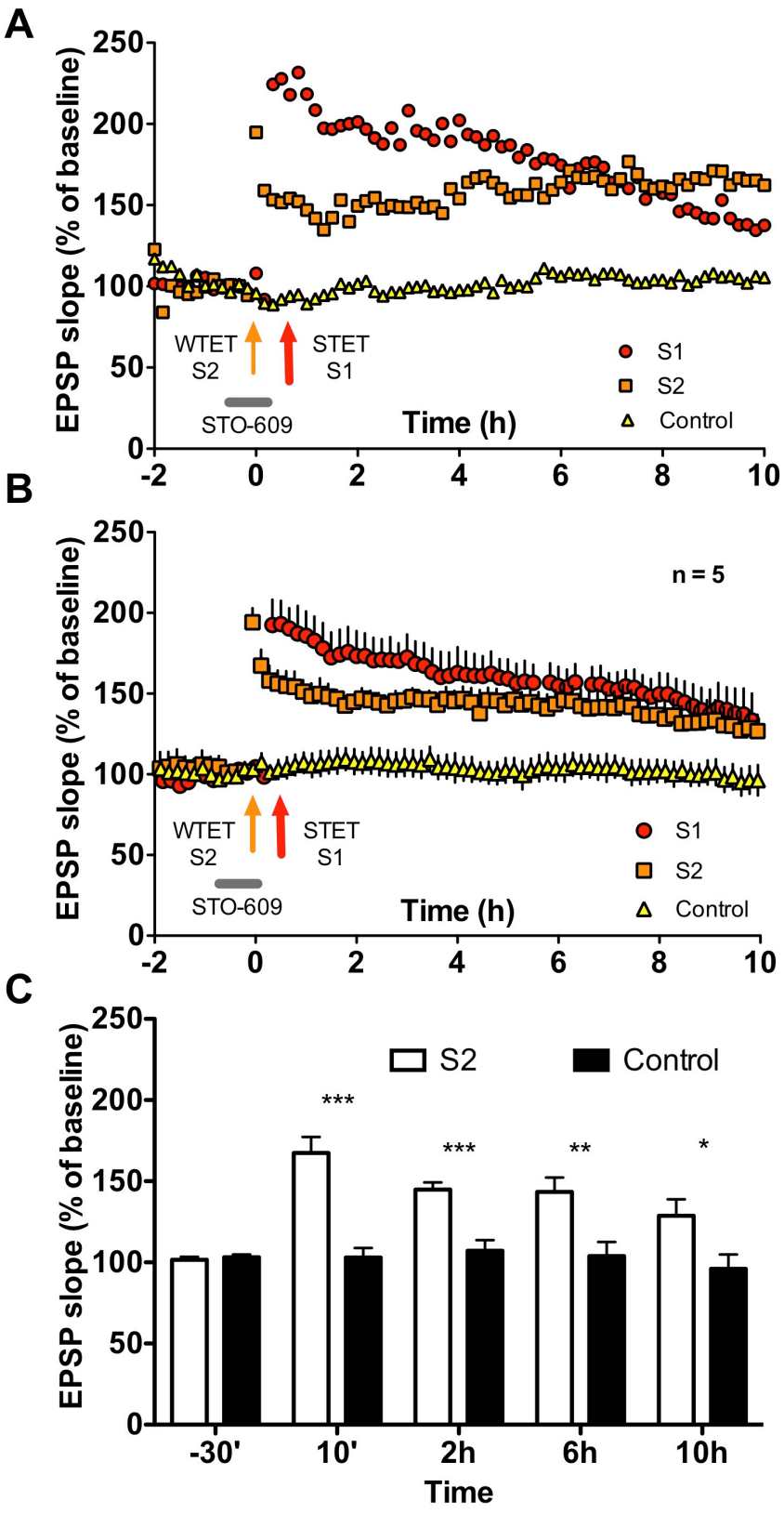


Figure 7.5 E-LTP is rescued into L-LTP with subsequent strong heterosynaptic stimulation even if the weak tetanus is given under the presence of STO-609.

A. An individual experiment showing a representative example of the rescue of E-LTP into L-LTP (S2) by heterosynaptic strong-tetanisation under the influence of 5 μM STO-609 (S1).

B. Grouped data for experiments in which L-LTP is seen in S2 (S2 vs. control pathway ($t = 2.7$, $p < 0.05$) and stable from 2 to 10 h (S2 2 vs. 10 h; $t = 2$, $p > 0.05$)) ($n = 5$).

C. Statistical comparisons were made between the weakly tetanised pathway (S2) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S2 and the control pathway were observed after 10 min ($t = 6.22$, $p < 0.001$), after 2 h ($t = 3.64$, $p < 0.001$) after 6 h ($t = 3.82$, $p < 0.01$) and after 10 h ($t = 2.98$, $p < 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
WTET
S2
STET
S1
STO-609
Time (h)
S1
S2
Control
B
n = 5
C
S2
Control
***
***
**
*
-30'
10'
2h
6h
10h
Time

7.3.3 5 μM STO-609 has no effect on a weakly tetanized pathway.

The effect reported in the previous section (Fig. 7.5) could be attributed to an enhancing effect of STO-609 on the synapses that received the 'weak tetanus'. To control for non-specific effects of STO-609 on the induction of LTP, a 'weak tetanus' was delivered to a set of synapses while under the influence of STO-609. The results confirm that STO-609 on its own is unable to rescue E-LTP into L-LTP (Fig. 7.7).

Figure 7.6 Representative fEPSPs waveforms from an individual 'STO-609 has no effect on weakly potentiated synapses' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 7.7

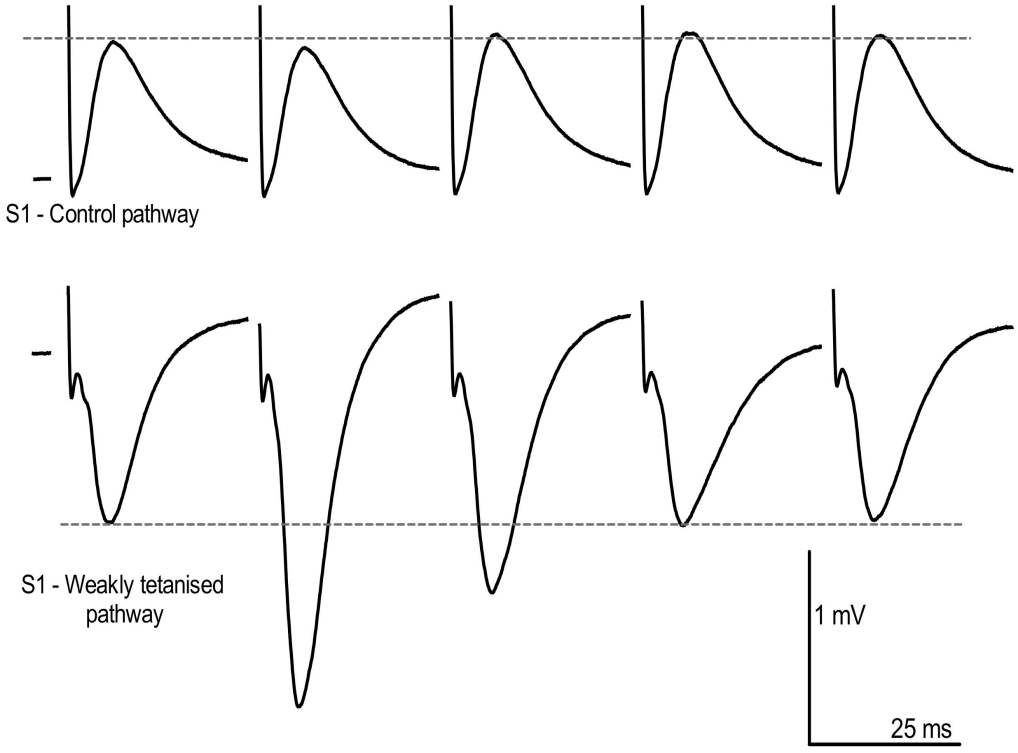
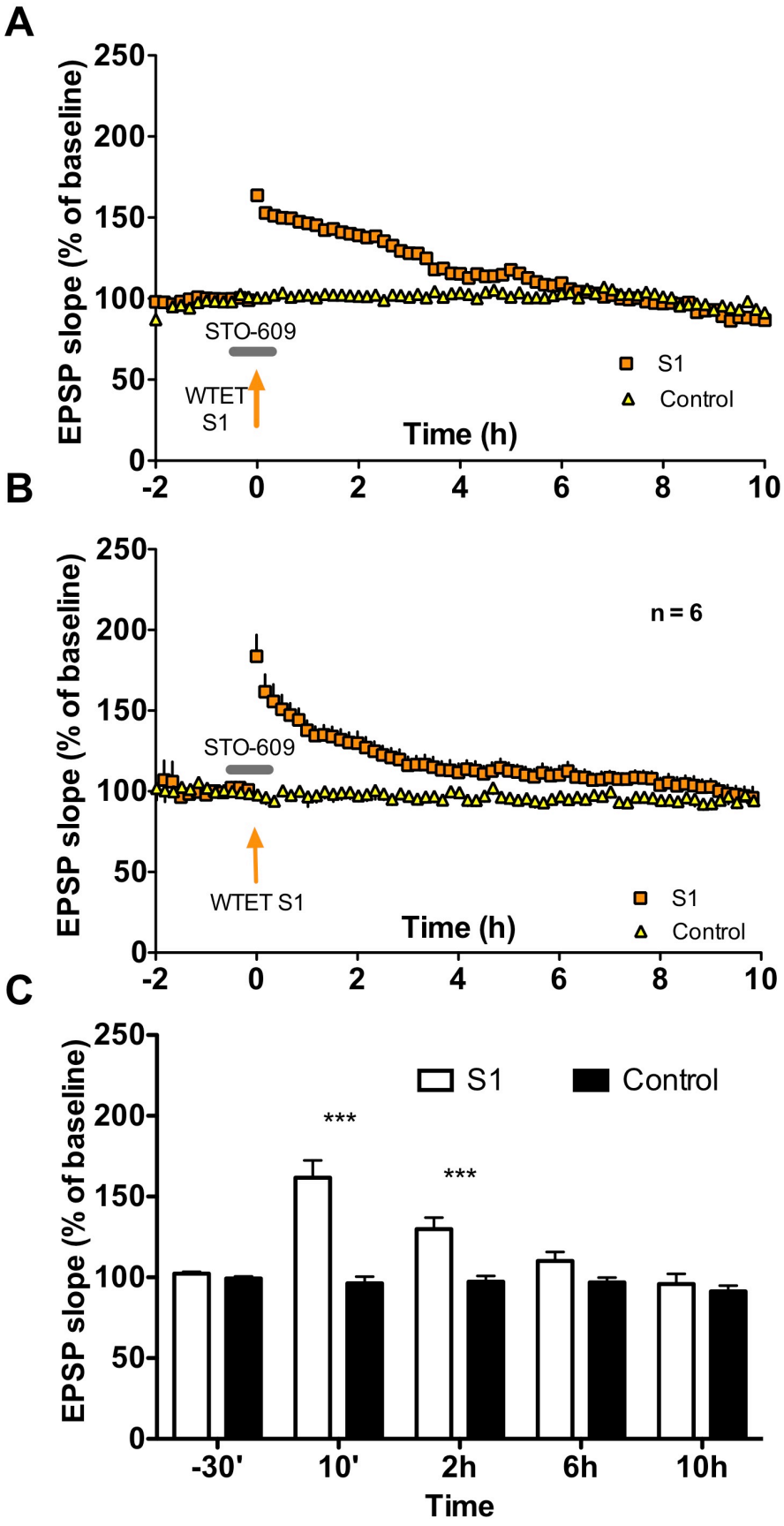


Figure 7.7 5 μM STO-609 lack of effect on weakly potentiated synapses.

A. An individual experiment showing a representative example of the rescue of E-LTP into L-LTP (S2) by heterosynaptic strong-tetanisation under the influence of 5 μM STO-609 (S1).

B. Grouped data for experiments in which only E-LTP is seen in S1 (S1 vs. baseline at 10 h; ($t = 0.65$, $p > 0.05$)) (n = 6).

C. Statistical comparisons were made between the weakly tetanised pathway (S1) and the non-tetanised control pathway (S2) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed after 10 min ($t = 8.56$, $p < 0.001$), after 2 h ($t = 4.25$, $p < 0.001$) but not after 6 h ($t = 1.73$, $p > 0.01$) or after 10 h ($t = 0.61$, $p > 0.05$).**


A
EPSP slope (% of baseline)
250
200
150
100
50
0
STO-609
WTET
S1
S1
Control
Time (h)
-2
0
2
4
6
8
10
B
EPSP slope (% of baseline)
n = 6
STO-609
WTET S1
S1
Control
Time (h)
C
EPSP slope (% of baseline)
S1
Control
***
***
-30'
10'
2h
6h
10h
Time

7.3.4 Another version of the 'PRP-block' protocol confirms the role of STO-609.

The classical version of the 'PRP-block' protocol as used in the original synaptic tagging experiments (Frey and Morris, 1997) involves the delivery of two 'strong tetanus' to two independent pathways with only one of them under the influence of the drug believed to have a role specific to the block of the availability of PRPs.We tested this protocol with STO-609 and report the rescue of the pathway stimulated under the influence of STO-609. This first pathway should show a decaying LTP (Fig. 7.3) but the second pathway, stimulated after the drug has been washed out, provides something capable of stabilizing the potentiation of the first (Fig. 7.9). The possible conclusions of this and the previous results are discussed below.

Figure 7.8 Representative fEPSPs waveforms from an individual 'Strong with STO-609 before Strong' experiment.

Individual fEPSPs from the tetanus (S1 and S2) recorded from *stratum radiatum* of the hippocampal slice. Individual waveforms are shown at different time points before and after the tetanus (the times are indicated under the waveforms). These waveforms are taken from the individual experiment shown in Figure 7.9

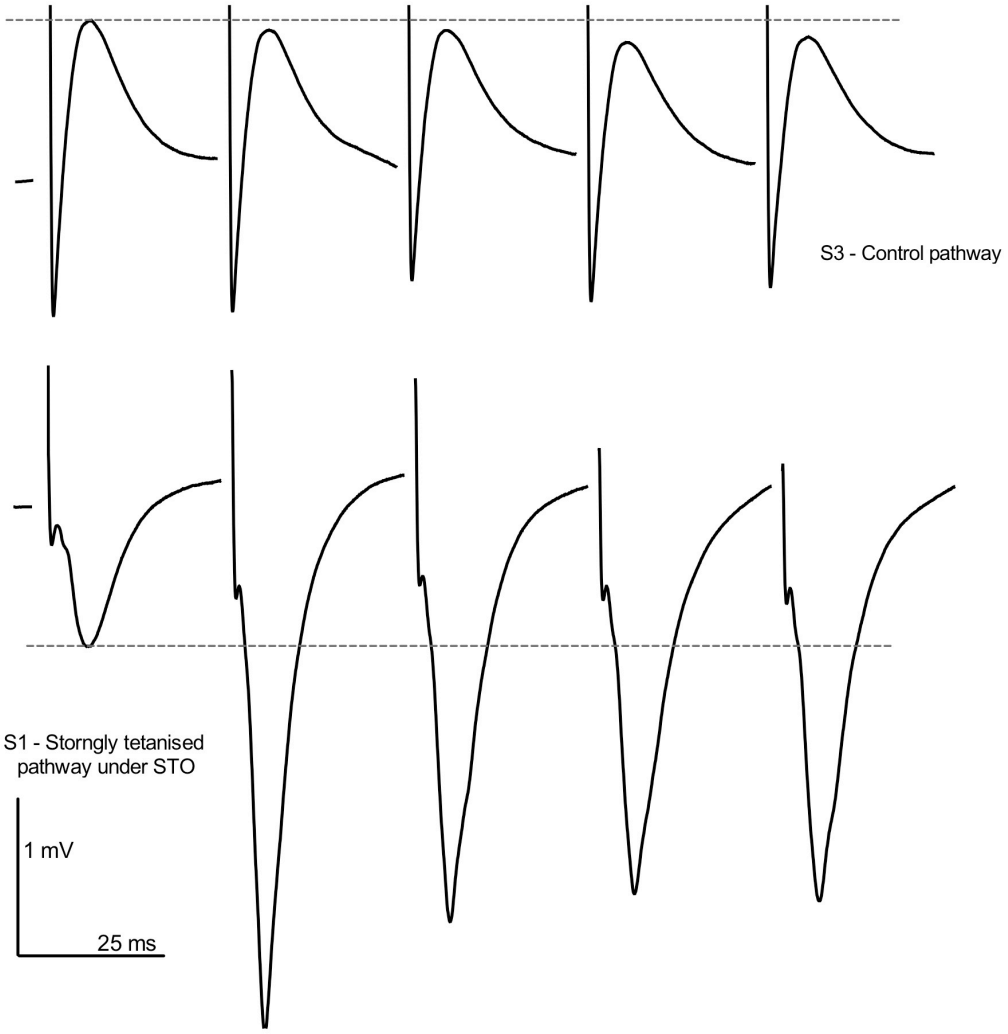


Figure 7.9 5 μM STO-609 blockage of L-LTP is rescued with subsequent strong heterosynaptic stimulation.

A. An individual experiment showing a representative example of the rescue of E-LTP into L-LTP in a strongly-tetanised pathway under the influence of 5 μM STO-609 (S1) by heterosynaptic strong-tetanisation (S2).

B. Grouped data for experiments in which L-LTP is seen in S1 (S1 vs. baseline at 10 h; ($t = 5$, $p < 0.01$)) ($n = 6$).

C. Statistical comparisons were made between the strongly tetanised pathway under the influence of 5 μM STO-609 (S1) and the non-tetanised control pathway (S3) at different times as indicated. Values represent the mean fEPSP slope (expressed as the normalised percentage change) measured at around each time point (averages of 10 measurements (i.e. 12.5 min (2.5 min per data point) before and after the time point). * indicates statistically significant difference with $p < 0.05$; ** indicates $p < 0.01$; * indicates $p < 0.001$ (unpaired students t – test, with Bonferroni correction for multiple comparisons). Significant differences between S1 and the control pathway were observed after 10 min ($t = 8.45$, $p < 0.001$), after 2 h ($t = 5.51$, $p < 0.001$) after 6 h ($t = 4.57$, $p < 0.01$) and after 10 h ($t = 3.89$, $p < 0.01$).**

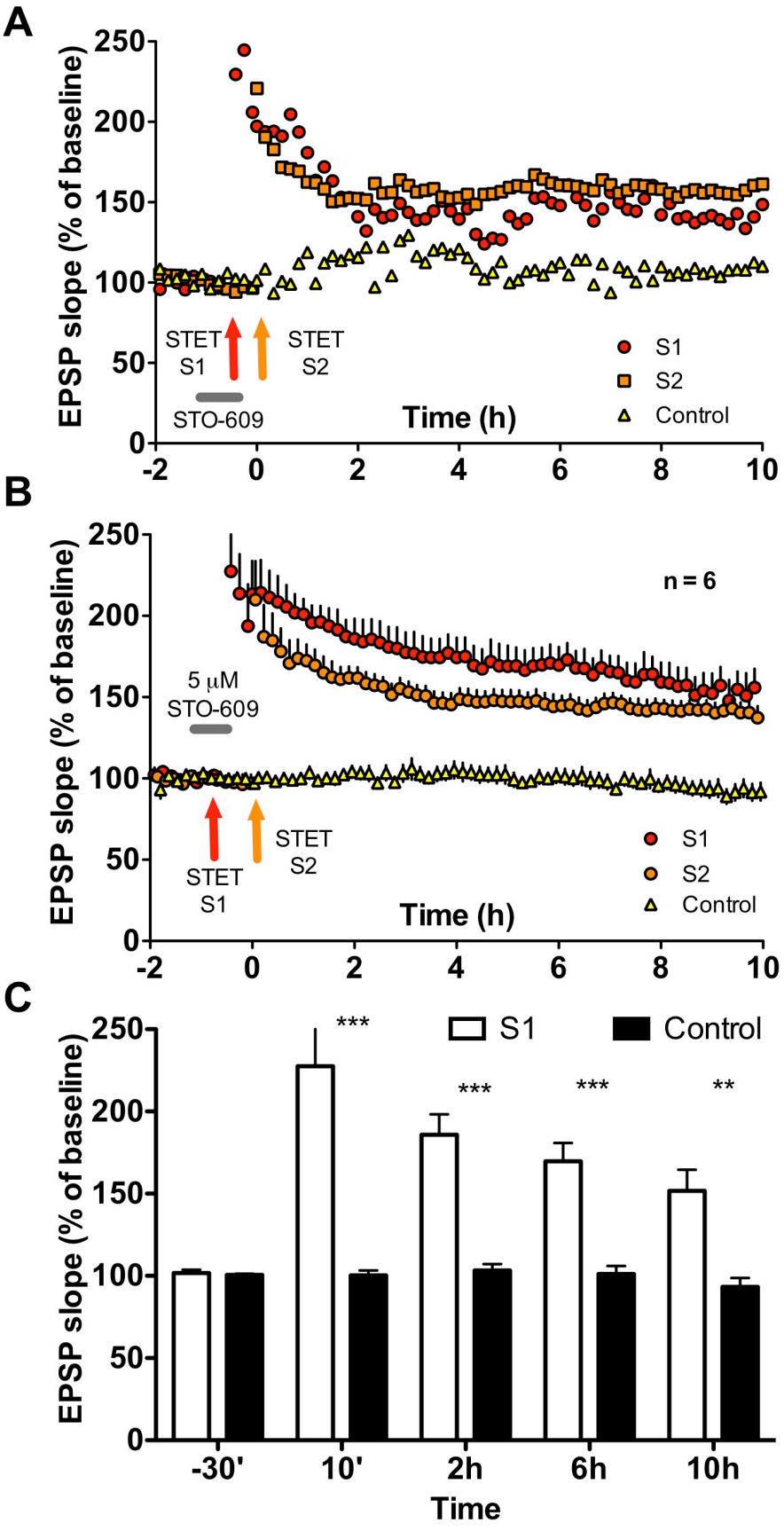
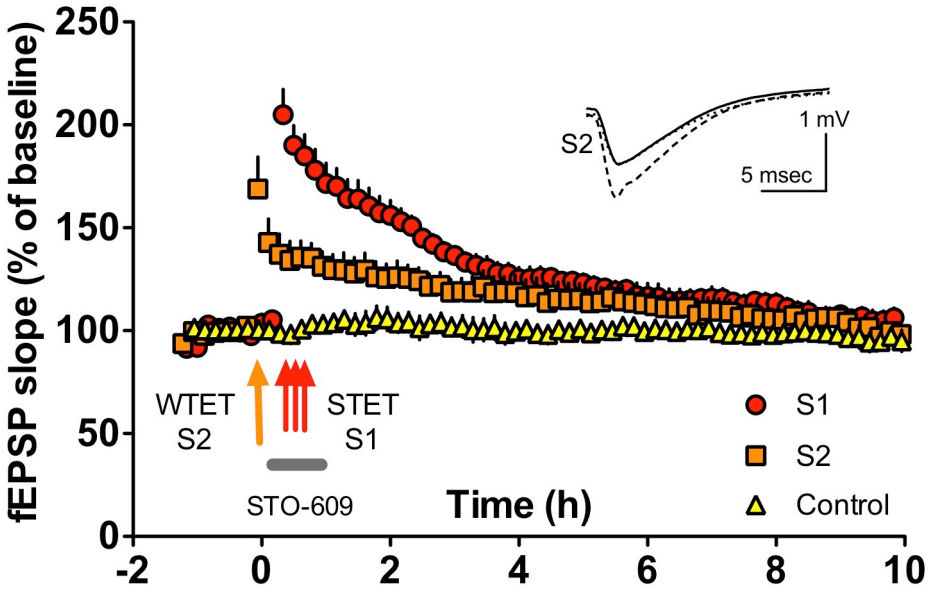

A
EPSP slope (% of baseline)
250
200
150
100
50
0
-2
0
2
4
6
8
10
STET S1
STET S2
STO-609
Time (h)
S1
S2
Control
B
EPSP slope (% of baseline)
n = 6
5 μM STO-609
STET S1
STET S2
Time (h)
S1
S2
Control
C
EPSP slope (% of baseline)
***
***
***
**
S1
Control
-30'
10'
2h
6h
10h
Time

Figure 7.10. STO-609 prevents the rescue of early-LTP into late-LTP.

If after the weak stimulation of pathway S2, STO-609 is applied during the strong tetanus to an independent set of synapses S1, the weakly potentiated synapses will show only early-LTP (S2 vs. baseline at 10h; t = 1.1, p > 0.05) (n = 6).



7.4 Discussion

The experiments described in this chapter (and chapter 6) have dissected the role of the CaM Kinase pathways in the maintenance of synaptic potentiation by dissociating between their synapse-specific and cell wide actions. Our findings and their interpretation emerge from our novel use of a long time course 3-pathway protocols (2 pathways tetanized, a 3rd non-tetanized control pathway). First, we confirm previous observations (Frey and Morris, 1997; Fonseca et al., 2004), that early-LTP induced at one set of synapses can be rescued into L-LTP if, within a short time-window (Frey and Morris, 1998b), L-LTP is induced at another set of synapses in the same CA1 neuronal population (chapter 5). Second, we show that a low concentration of KN-93 at the time of tetanization blocks L-LTP (Figs 6.7 & 6.9) and does so by interrupting a pathway-specific process that cannot be overcome by tetanization of a second independent pathway (Fig 6.3 & 6.5). A higher concentration of KN-93 also blocks L-LTP, but without this specificity (Fig. 6.11 & 6.13). This can be explained by cell culture data that reveals differential concentration-response actions of KN-93 for synapse-specific and cell-wide actions (Supplemental data Fig. S1). Finally, in this chapter, we establish that STO-609 also blocks L-LTP but does so by interrupting a cell-wide process that can be overcome by tetanization of a separate pathway.

7.4.1 CaMKK activity is necessary for PRP availability but not tag setting.

Does STO-609 block L-LTP through a specific effect on the availability of PRPs? To test this hypothesis, we used a 'tag block' test (Fig. 1.4) which involved STO-609 being present during the weak tetanization of one pathway (S2) but washed it out before strong tetanization was applied to pathway (S1). In this 'weak-before-strong' protocol, we observed a rescue of early-LTP into L-LTP on the weakly stimulated pathway S2 (Fig. 7.5). This was not due to some cryptic 'potentiating' effect of STO-609 on a weakly tetanized pathway as giving this drug during weak tetanization of a single pathway was without effect on its decay to baseline (Fig. 7.7). Thus, STO-609 blocks L-LTP but it does not block tag setting, implying that inhibition of the CaMKK-dependent pathway limits the synthesis or availability of PRPs. We tested

this directly using a standard ‘strong-before-strong’ STC protocol in which STO-609 was present during one of the two strong tetanizations. In keeping with our interpretation, pathway S1 showed L-LTP (Fig. 7.9). Supplementary experiments using a weak-before-strong protocol with STO-609 present during strong tetanization revealed no L-LTP on the weakly stimulated pathway, in keeping with this interpretation (Supplemental Fig. 7.10).

Chapter 8 Behavioural Tagging: Exploration Preceding Encoding Enhances the Retention of One-trial Place Memory.

8.1 Introduction

Following the electrophysiological results presented in chapters 3 to 7, the synaptic plasticity and memory hypothesis (Martin et al., 2000b) predicts that there should be some way of translating the STC hypothesis into a behavioural counterpart. If synaptic strength is in some way a correlate for memory formation, weak and strong tetanisations could be translated into behaviour as weak and strong memories. Although the behaviour may be more complicated with different brain structures involved, we know that the stabilization of memories requires the synthesis of new proteins around the time of encoding (Davis and Squire, 1984). The question becomes then, do the heterosynaptic interactions described by the STC hypothesis have a correlate in behaviour? Could a weak memory last longer if the same cells that encode it have received a strong encoding event at the appropriate time?

8.1.1 Novelty as the tool to make PRPs available to synapses encoding a weak memory.

In animal studies, one serious problem when trying to encode two memories in a short time interval is the two of them interfering with each other. Our laboratory has faced this problem when trying to teach rats the location of hidden platforms in the Morris watermaze in two separate rooms with a different arrangement of spatial cues. In order to avoid the problem of two memories interfering with each other, we decided to focus on the fundamental element that the strong electrical stimulation provides to the weakly tetanised synapses in the framework of the STC hypothesis (i.e. the plasticity related proteins (PRPs)). If the PRPs are the missing ingredient for E-LTP to be stabilized into L-LTP, then behaviourally, a short-lasting memory could benefit from the availability of PRPs. The goal then would be making these PRPs available to the cells encoding the short-lasting memory. If there was a way of making the PRPs available without presenting the individual with a second strong

encoding event, then the problem of interference between two memories that rely on the same pool of neurons should be avoided. Fortunately, the literature provides us with some ways of upregulating the synthesis of PRPs via a behavioural manipulation.

We sought to schedule an event known to induce the synthesis of a plasticity-associated mRNA in CA1 neurons - spatial exploration of a novel environment (Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004) - shortly before the memory encoding of the location of food in a one-trial place memory task ('event arena' - see Methods chapter 2) that ordinarily shows forgetting over 6 h (Bast et al., 2005). This chapter describes a series of behavioural experiments that pursue the lengthening of the persistence of a memory by pairing its encoding with an event capable of upregulating PRPs.

8.2 Methods

The general methods concerning the 'event arena' one trial match to place protocol are summarized in chapter 2. What follows is a description of methods particular to this chapter.

Twelve male Hooded Lister rats (250-430g) were housed individually in stainless steel cages. The rats were kept at 85% of free-feeding body weight. All rats were maintained on a 12 h light/12 h dark schedule, with testing occurring in the light phase. The animals were trained and tested in a one-trial place memory task. Trials consisted of a memory encoding 'sample' phase and a retrieval 'choice' phase. In the sample phase, the rat spent 30 s in the startbox before the door opened allowing entry into the arena where a single, rewarded sandwell was uncovered. The rat was given 60 s to eat the 0.5 g pellet before being returned to its home cage for a 5 min memory delay. In the choice phase, the rat spent 30 s in a different startbox before being let out into the arena. The same sandwell again contained food, but was now presented along with four other unrewarded wells located in other positions. Thus, the animal could use one-trial place memory and a win-stay rule to most efficiently retrieve the 0.5 g reward. Start boxes for the sample and choice trials, were varied to promote the use of allocentric place memory, and the locations of the rewarded and unrewarded wells were changed daily in a pseudo-random fashion. Counterbalancing ensured that a particular sandwell location was as often near or far from a startbox on a given day. To help prevent the use of smell to find the rewarded sandwell, the wells consisted of 95% sand and 5% crushed food pellets. Only one sample-choice trial was given on each day for each animal. Performance was measured in terms of latency and the number of errors made before reaching the rewarded well. After 33 days of training, performance was consistently above chance (mean errors < 2, where an error was defined by digging in an incorrect well prior to the correct well), and the main experiment then conducted.

The exploration of a novel box was analogous to a procedure that had been shown to induce upregulation of Arc mRNA in hippocampal cells (Vazdarjanova and Guzowski, 2004). This consisted of 5 min exploration in a 1 m x 1 m square Perspex (Plexiglas) box that, importantly, was placed inside the event arena. Thus, the

extramaze cues were unchanged and the intramaze cues moved only a short distance towards the side of the arena to accommodate the box. The memory testing protocol involved the rats being tested successively in three separate conditions using a counterbalanced within-subjects design (Fig. 8.1). The 'sample-choice' trial-pairs were given exactly as in training, except for three key differences: 1) the *memory interval* between sample and choice was changed to either 20 min or 6 h; 2) the choice trial was a *probe* trial in which none of the wells contained the reward; and 3) all animals spent the interval between sample and choice in the *animal room*. Two regular training days were given between each testing day. Thus, to complete three conditions, there were a total of 9 days, including 2 days prior to the first probe trial.

The data monitored during training were latency (s), number of first choices of the correct well, and errors (wrong choices) before reaching the correct well. During non-rewarded memory test trials, the primary data measure was time spent digging in each of the five wells during the 60 s in the arena on a probe test. All probe tests ended with 0.5 g reward placed into the correct well to prevent extinction. Time spent digging in the correct and novel wells was converted into percentage dig time in the 60 s of exploration, and for purposes of comparison the average percentage dig time in novel wells was computed (i.e. the sum of dig time in all novel wells divided by four for each animal).

8.3 Results

8.3.1 Memory for correct location lasts 6h in the exploration condition

All 12 rats learned to run from the startboxes to the single open sandwell during the daily sample phase, and to visit this location preferentially relative to the other four of the five sandwells open during the choice phase by the end of training (data not shown). Memory for location was tested using non-rewarded probe tests that measured the proportion of time spent digging at the sample sandwell relative to the four other sandwells 20 min or 6 h after encoding (Figure 8.1). The differences seen in this experiment when looking at the percentage of dig time spent in the correct well (One-way ANOVA $F_{2,22} = 8.72$, $p < 0.01$) show that rats spent significantly more time digging in the correct well at a 20 min memory retention interval than at 6 h controls (Bonferroni $t = 4.139$; $p < 0.001$) indicating a decline in memory over this time period (Fig. 8.2). There was a strong but non-significant trend (under the conditions of the ANOVA) to an improvement in the memory performance after 6h if the animals had been allowed to explore the novel box for 5 min before the encoding of the correct location (diff. between novelty and control Bonferroni $t = 1.59$, $p > 0.05$).

Behavioural assessment of memory performance has to be done with care to avoid the problems of ceiling and floor effects. In this task, chance performanca is 20% (5 wells equally explored without a preference for the correct well). The mean performance of the 6 h control group is 23.73 %. It could well be that it this is the lowest performance that we can obtain and that comparing this floor performance to the 6 h treatment condition would not reveal an enhancing effect of the treatment.

Consequently, instead of comparing the groups between themselves, we can ask the question of whether the animals are capable of performing above chance after a given retention delay. That is, if we take 20 % dig time in the correct well as chance performance (20% dig time for 5 wells), then, while the performance after 6 h did not differ from chance ($t = 1.45$; $p > 0.05$), the performance after exploring the boxed environment is well above the chance level ($t = 3.47$, $p < 0.01$), as is the performance

in the shorter 20 min delay test ($t = 4.49$, $p < 0.001$). Thus, the exploration of a box for 5 min, 20 min before the encoding of the location allows for that memory to last for at least 6 h (Fig. 8.2A).

The results presented so far make use of the measurement of the percentage of time spent digging in the correct well with respect to the total time that the rat spends digging during the 60 s probe trial. There are other potentially useful parameters that traditionally have been used to assess memory performance. Briefly, the percentage of animals choosing the correct well as the first choice for digging during the probe trial (Fig. 8.2B) is a single value (no population measures) that as seen in Figure 8.2A nicely follows the percentage dig time.

Another parameter used regularly in behaviour is the number of errors that the rat makes before digging in the correct well. In this task however, the rats seem to adopt a tendency to stop in every well they encounter. Consequently, while the percentage of time spent digging in the correct well is a good measure of memory performance, even animals that dig mostly in the correct well, may have reached that location while stopping and briefly digging in novel, incorrect wells. The graphical representation of the errors before reaching the correct well confirms this behaviour (Fig. 8.2C)

Fig. 8.1. Experimental design to test the effect of the exploration of a novel environment on the persistence of a spatial memory.

As described in the methods section of this chapter (and in chapter 2), 12 rats were trained to remember the location of food reward in the event arena. Although the training consisted of a retrieval phase where the correct well contained food (not shown here), the probe tests used to assess memory performance consisted of an encoding phase with one rewarded well while the retrieval phase had no reward in any of the 5 wells at the disposal of the rat. The probe test consisted of 60 s during which the location and duration of the animals' digging times was recorded. This probe test was carried out 20 min (A) or 6 h (B & C) after the encoding of the spatial location. C, The "novel box" manipulation consisted in a 5 min exploration period, in the event arena, inside a square-plexyglass box, 20 min before a regular encoding phase.

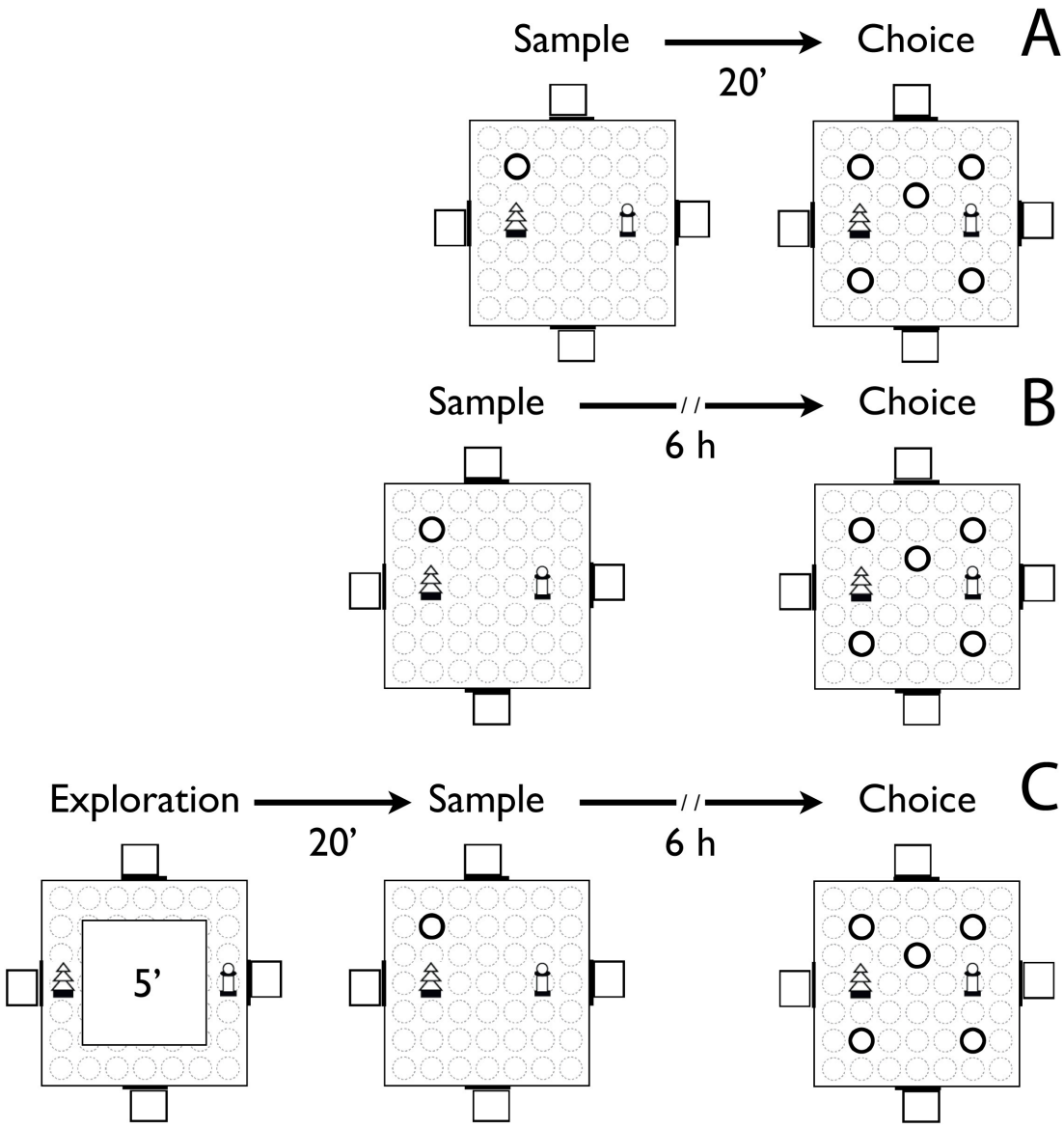
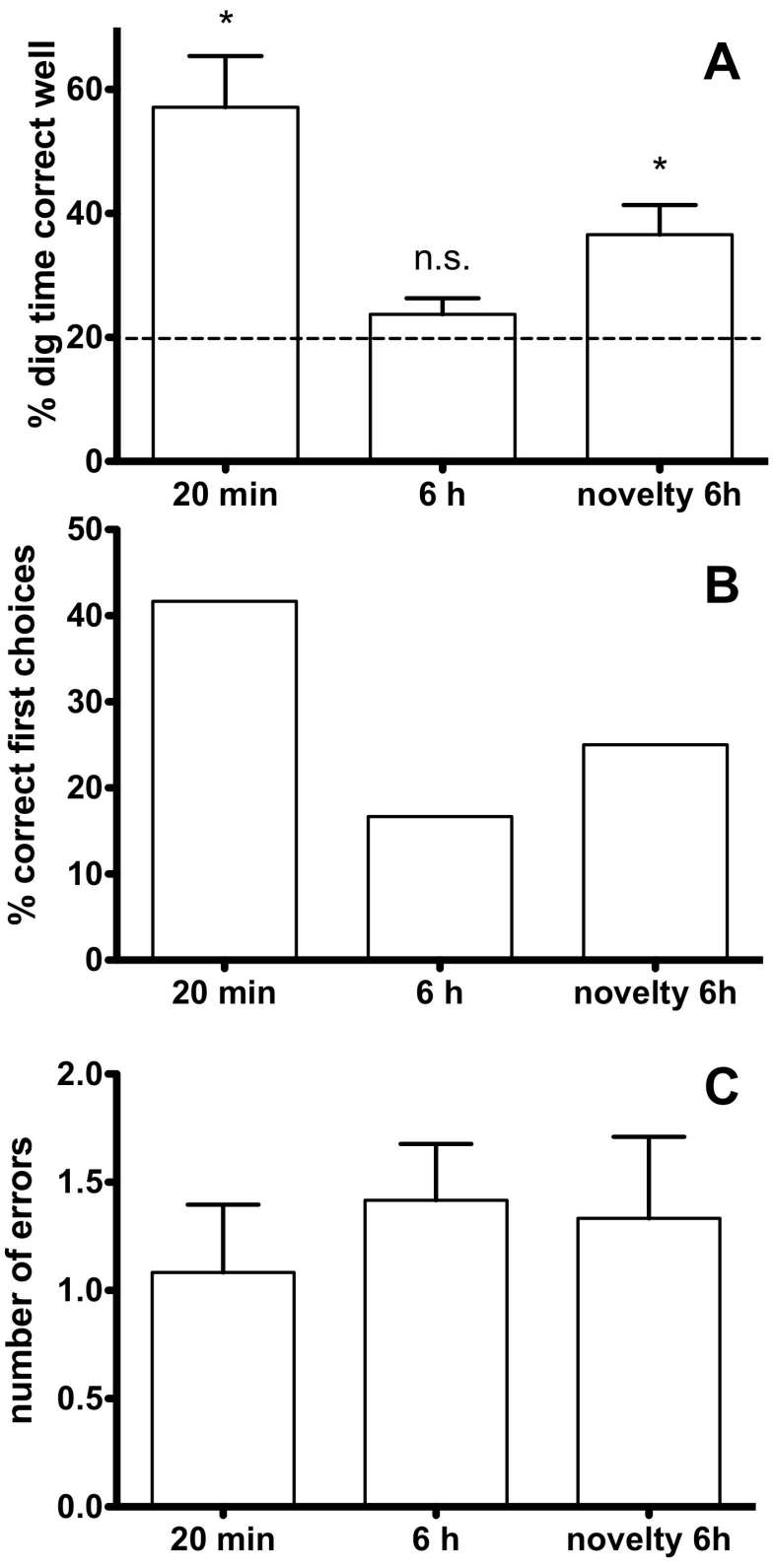

A
Sample
20'
Choice
B
Sample
6 h
Choice
C
Exploration
20'
5'
Sample
6 h
Choice

Fig. 8.2. Novel-exploration rescues memory after 6 h.

A. Compared to chance level (20% dig time), the animals were capable of showing a preference for the correct well after 20 min ($t = 3.47$, $p < 0.01$) but not after 6 h ($t = 1.45$; $p > 0.05$). However, the exploration of the novel box 20 min before the encoding, allowed these same animals to show a statistically significant preference for the correct well even after the 6h ($t = 4.49$, $p < 0.001$).

B. The graph depicts the percentage animals that chose the correct well as their first choice for digging, between the three conditions under study.

C. The variability in the number of incorrect wells that the animals dug before reaching the correct well (i.e. number of errors) is too high to detect any differences between conditions (One-way ANOVA $F_{2,22} = 0.23$; $p > 0.05$).


A
% dig time correct well
60
40
20
0
*
n.s.
*
20 min
6 h
novelty 6h
B
% correct first choices
50
40
30
20
10
0
20 min
6 h
novelty 6h
C
number of errors
2.0
1.5
1.0
0.5
0.0
20 min
6 h
novelty 6h

8.3.2 Variables behind the enhancement of memory by novelty exploration: is there a need for novelty and external cue overlap?

The exploration of a novel box allowed for a spatial memory to be maintained for longer than in control conditions. However, is it the exploration of the box or the novelty that has this effect? Are the two dissociable?

To test this, the animals underwent a series of retraining days in which they had a chance to familiarize themselves with the square box seen in the previous experiment. The two new boxes (triangular and a triangular prism) were used to reproduce the effects of novelty (Fig. 8.3).

Also, is this a case of a behavioural manipulation that strengthens the memory or is the maintenance of the memory lengthened. In an attempt to see whether the treatment was having an effect at the short delay interval, we introduced an additional condition where the animals would experience the treatment (novelty-exploration) before the sample phase, but instead of being tested after 6 h, they would be tested after 20 min (Fig. 8.3).

Finally, there was an attempt to find out whether the exploration had to be in the same room as the behavioural testing or if a similar enhancement could be obtained if the exploration took place in a different environment. With this in mind, a condition was added where the exploration would take place in a separate room, adjacent to the behavioural arena but not seen by the animals before (Fig. 8.3).

The results are presented graphically in Figure 8.4 and when working with the percentage of dig time spent in the correct well show overall differences between conditions (One-way ANOVA $F_{5,55} = 4.99$, $p < 0.001$). There is no effect of novelty-exploration at the short interval of 20 min when compared to control condition at the same retention delay (Bonferroni $t = 0.03$, $p > 0.05$). As discussed below, this result is not very informative because it leaves the possibility of a ceiling effect in performance (i.e. the animals's performance is cannot improve above a certain level) occluding the effect of the treatment.

Interestingly, there is no difference between control 6 h delay condition and the other 6 h conditions (One-way ANOVA of the subset of conditions tested after the 6 h delay $F_{3,33} = 1.04$, $p > 0.05$). Looking at the graphs there seems to be a trend

towards improvement and the first choice and error data may also suggest this (Figure 8.4 B & C). Further analysis of the numbers of errors (Fig. 8.4 C) shows significant differences in the 6 h conditions (One-way ANOVA $F_{3,33} = 2.91$, $p < 0.05$) but none of the pairwise comparisons reveal statistical significance.

This set of experiments could not distinguish between familiar and novel exploration and it also fails to answer whether the behavioural manipulation has to be performed in the same environment as the encoding of the memory. How close is the exploration of the novel box to the PRP-upregulating event that would fit in a behavioural model of the STC hypothesis?

A more reliable version of this task, together with pharmacological manipulation was necessary to answer these questions (chapter 9).

Fig. 8.3 Experimental design to test the importance of familiarity and the overlap of external landmarks on the effect of the exploration of the novel box.

The goal of this set of experiments was to test three things:

A. **What is the effect of the novel box exploration on the short-term (20 min delay) memory?**

B. **Is the effect at 6 h lost after the familiarization with the box?**

C. **Will a new box still enhances the memory at 6 h?**

D. **Does the novel exploration need to take place in the same environment as the memory task?**

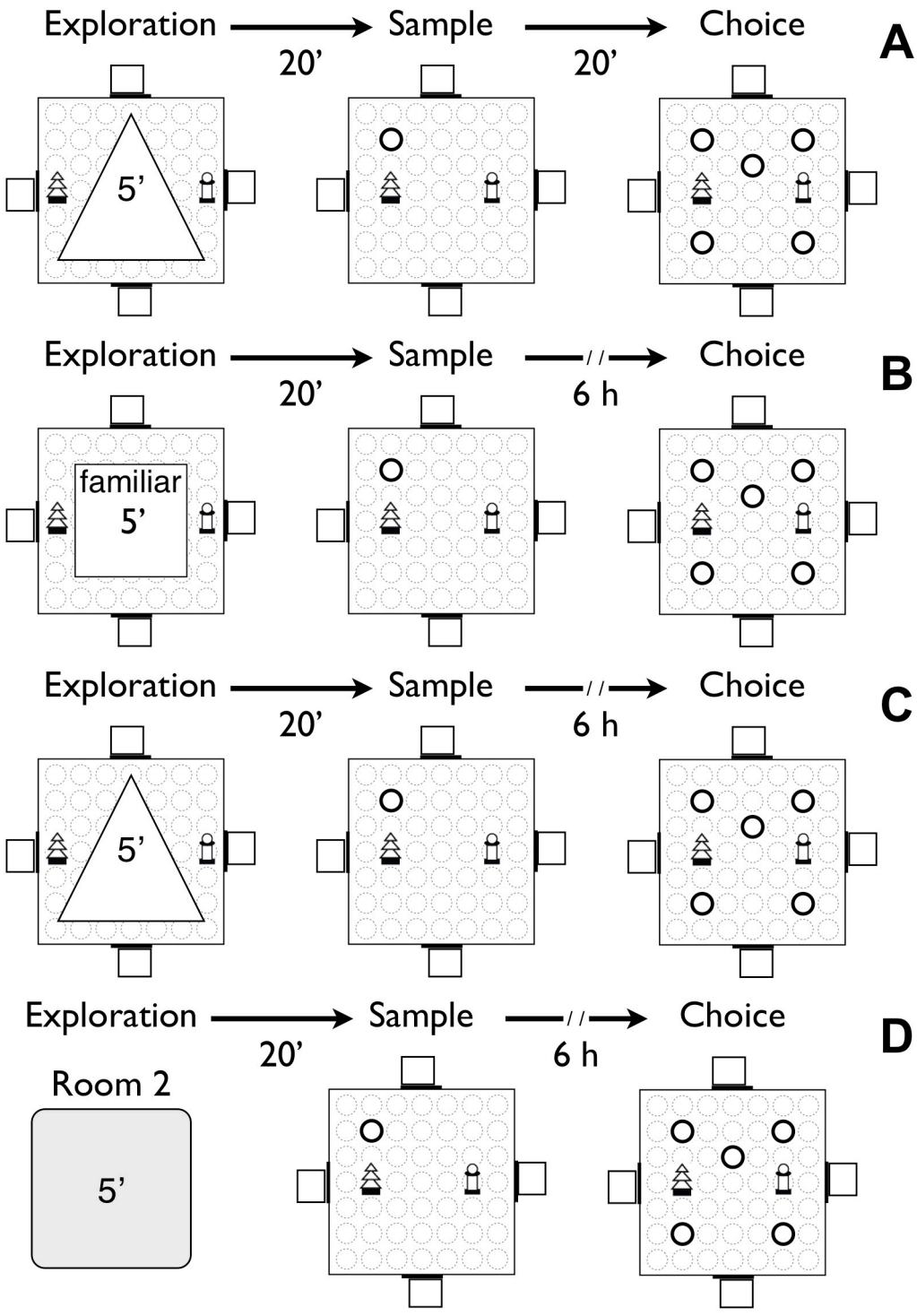
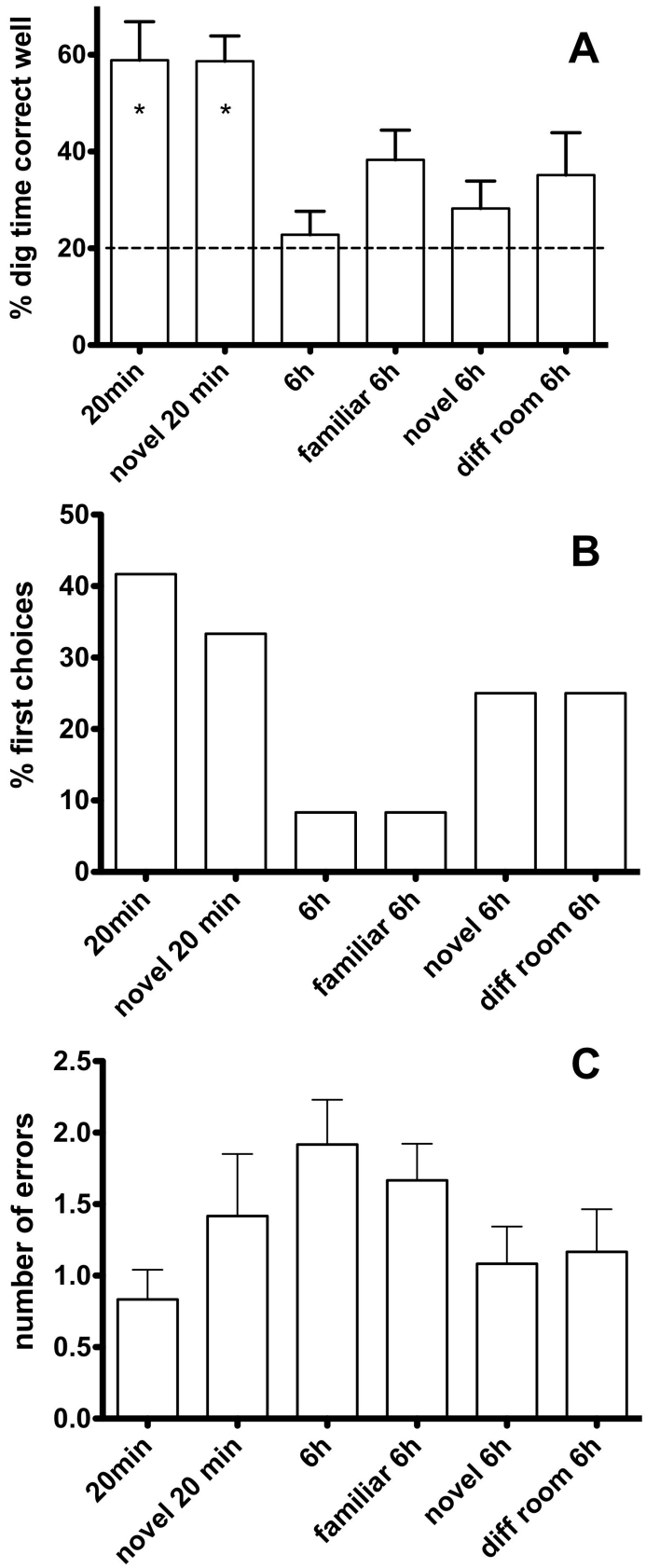

Exploration
20'
Sample
20'
Choice
A
5'
Exploration
20'
Sample
6 h
Choice
B
familiar
5'
Exploration
20'
Sample
6 h
Choice
C
5'
Exploration
20'
Sample
6 h
Choice
D
Room 2
5'

Fig. 8.4. Experiment Two Results: Percentage Dig Time (A), Percentage First Choice (B) and Error Performance Score (C).

A. Comparing the percentage dig time in the 6 conditions under study reveals overall differences (ANOVA $F_{5,55} = 4.99$; $p < 0.001$). However, the comparisons of interest fail to find significant differences (under Bonferroni corrections). There is no difference in memory retention after a 20 min delay, measured as percentage dig time, when the same animals have explored a novel environment or not ($t = 0.02$, $p > 0.05$). At the longer delay of 6 h, there was no difference in performance compared to the control condition when the animals explored a familiar environment ($t = 1.61$, $p > 0.05$), a novel environment ($t = 0.56$, $p > 0.05$) or when the exploration took place in a novel environment ($t = 1.28$, $p > 0.05$). An independent comparison of the performance in each condition with the 20% chance level of this task confirms the lack of memory retention at 6 h (data not shown).

B. The graph depicts the percentage of trials where the animals chose the correct well as their first choice for digging, between the six conditions under study.

C. The variability in the number of incorrect wells that the animals dug before reaching the correct well (i.e. number of errors) is too high to detect any differences between conditions ($F_{5,55} = 1.79$, $p > 0.05$).


A
% dig time correct well
60
40
20
0
*
*
20min
novel 20 min
6h
familiar 6h
novel 6h
diff room 6h
B
% first choices
50
40
30
20
10
0
20min
novel 20 min
6h
familiar 6h
novel 6h
diff room 6h
C
number of errors
2.5
2.0
1.5
1.0
0.5
0.0
20min
novel 20 min
6h
familiar 6h
novel 6h
diff room 6h

8.4 Discussion

The first key finding of this behavioural study is that the unexpected exploration of a novel environment in the same context as that in which repeated one-trial place memory was tested daily, prevented the decay of the memory for the location of food (Fig 8.1 & 2).

The main finding is consistent with recent evidence that novelty exploration enhances the induction and maintenance of LTP (Li et al., 2003; Davis et al., 2004; Kemp and Manahan-Vaughan, 2004). Further, while no pharmacological tests were conducted in this study, our results are consistent with the idea that novel stimuli might act via neuromodulatory pathways to produce a hippocampal state that is conducive to the persistence of LTP (Meeter et al., 2004; Lisman and Grace, 2005). Such a state might be brought about by activation of dopamine D1 receptors that, via several different signalling cascades, can trigger the synthesis of proteins necessary for L-LTP. Always within the framework of the STC hypothesis and the 'synaptic plasticity and memory' hypothesis, more plasticity-proteins would be available to the tagged synapses of the cell, and a greater proportion of them could then be stabilized to L-LTP (Fonseca et al., 2004; Sajikumar and Frey, 2004a). The rationale behind using exploration in the clear square box in the event arena as a 'treatment' was Vazdarjanova (Vazdarjanova and Guzowski, 2004) observation that exploration in two similar, novel environments causes greater cellular overlap of Arc mRNA in area CA1 than occurs with two dissimilar environments. The goal was to maximize the probability that those cells in which an upregulation of protein synthesis occurs in response to novelty exploration could be the 'place cells' that coded for the location of the food. However, having established a behavioural protocol that can yield long-term memory from an encoding protocol that normally only produces short-term memory, future experiments should compare the effectiveness of out-of-context novelty treatments that might trigger neuromodulatory transmission in a diffuse way with a novel treatment, such as the present one, that is conducted *in situ* and so more likely to upregulate protein synthesis in an appropriate sub-population of place cells.

In conclusion, the behavioural protocol developed in this set of experiments offers evidence that the persistence of a normally decaying memory can be enhanced by a

treatment known to up-regulate protein synthesis and the persistence of LTP. In conjunction with suitable *in vivo* pharmacology (Chapter 9), the task used in this chapter could provide a means for studying the functional significance of heterosynaptic plasticity, as described by the STC hypothesis, in learning and memory.

Chapter 9 Behavioural Tagging: Spatial exploration prolongs the maintenance of a spatial memory in a dopamine and protein synthesis dependent manner.

9.1 Introduction

After the positive results described in Chapter 8, the one-trial match to place task was slightly modified to increase the consistency of the animals' performance so longer experiments could be used to elucidate the mechanisms behind this potential correlate between the STC hypothesis and behavioural memory. These experiments had the objective of expanding our knowledge behind the mechanisms responsible for the enhancement in memory retention described in the previous chapter.

As explained in Chapter 1, novel experiences can affect the induction of synaptic plasticity, LTP or LTD (Xu et al., 1998; Kemp and Manahan-Vaughan, 2004) and this effect is dependent on activation of D1/D5 receptors in the area CA1 of the hippocampus. This chapter aims to explore the role of dopamine and the synthesis of PRPs in the context of behavioural tagging.

9.1.1 Dopaminergic action and requirement for protein synthesis in behavioural tagging

Hippocampal dopamine depletion impairs (and D1 agonists enhance) certain types of hippocampus dependent learning: spatial memory in watermaze (Gasbarri et al., 1996), spatial memory in watermaze for aged-impaired rat (Hersi et al., 1995) and mice (Bach et al., 1999).

What is the role of dopamine in the enhancement of memory described in Chapter 8? Will dopamine antagonists have the same effect as in the behavioural tagging described in a weak training in inhibitory avoidance task (Moncada and Viola, 2007)? The experiments in this chapter investigate whether D1/D5 receptor activation is necessary in a spatial task that has substantial evidence for hippocampus dependency and whether a complete rescue of memory impairment is possible by a novel experience. Therefore, we used the one-trial match to place spatial learning task in the event arena to examine 'behavioural tagging' and its mechanism in the

hippocampus. To this end, exploration in a novel box was used in conjunction with the encoding of a spatial location in the event arena.

Also, immediate early gene expression in the hippocampus has been widely associated with spatial learning (Vazdarjanova et al., 2006). It is likely that the novelty-induced gene expression and protein synthesis in the hippocampus can facilitate spatial memory maintenance. If so, the inhibition of protein synthesis in the hippocampus would be predicted to impair the facilitatory effect of novel exploration.

All the experimental protocols used in these experiments are depicted graphically in figure 9.1.

9.2 Methods

16 rats were trained in the event arena with one-trial learning task (described in Chapter 2, section 2.3). Only 11 rats participated in the experiments involving the infusion of drugs (figures 9.3 onwards). This section describes a few modifications on the standard protocol that ensured consistent performance of the animals consequently allowing multiple experiments on the same subset of rats.

Adult Lister-Hooded rats were mildly food deprived and maintained at 90% of normal weight. Rats were habituated with digging the sand wells for flavoured food pellets. The training in the event arena (Chapter 2 Fig. 2.7) consisted of 1 encoding phase in which the rats were allowed to entered the arena from one of the four start box to obtain pellets from one baited well. The rats were trained to carry and eat the pellet in the start box. Once the rat successfully collected and ate three pellets, they were returned to the home cage. Half an hour later, they were put in the start box for 'reinforced' probe test in which four un-baited wells and one baited well at the same location that appeared at the encoding phase. The start box location, baited well locations and un-baited well locations were counterbalanced across rats and training days. The probe test results showed that the number of errors that the rats made before finding the pellet at the correct well significantly reduced across the 17 training days (supplementary Fig. S4).

SCH23390 hydrochloride (Tocris, UK) was dissolved in sterile normal physiological saline and kept in frozen aliquots (500 µl) until usage. SCH23390 final concentrations of 1 mg/ml or 3.3 mg/ml were used in this study. Anisomycin (Sigma-Aldrich, UK) was dissolved in 1N HCl, diluted with sterile normal physiological saline and adjusted to pH 7.4 with 1N NaOH to produce a final concentration at 125 µg/µl. d-AP5 (Sigma-Aldrich, UK) was dissolved in normal physiological saline and adjusted to pH 7.4 to produce a final concentration at 5.9 mg/ml.

An infusion pump was used for bilateral infusion at a flow rate of 0.25 µl/min. Microsyringes (5 µl, SGE, Australia) were mounted on the pump and connected with injection cannulae (33 gauge, 0.5mm beyond the guide) using flexible polyethylene tubes. During infusions, the rats were restrained lightly with a towel. The injection

cannulae were left in the guide cannulae for 1 minute after the infusion. Once the injection ended, the dummy cannulae were placed back into the guide cannulae.

The maximal and minimal position of the cannulae at selected coronal planes through the hippocampus has not yet been reconstructed since the brains are awaiting further processing by our collaborators.

This project was done in collaboration with Dr. Szu-Han Wang who ran most of the behavioural tasks, for which I am very grateful . I contributed by training the animals, with the cannulae implantation, preparing and infusing the drugs to the animals, and planning and running some of the behaviour in the early and late phases of the experiment.

9.3 Results

9.3.1 Exploration lengthens memory retention

After the rats acquired the task, we first looked at the retention of a weak encoding event (i.e. 1 pellet) and asked whether exploration in a novel box can enhance the memory retention. The probe tests revealed that the rats maintained good 1-pellet memory for 30 min but not for 24 h (Fig. 9.2A). However, if the rats explored a novel box for 5 min after the 1-pellet encoding, they preferentially dug at the correct well where the pellet was baited the day before (Fig. 9.2B), suggesting the novel box experience can enhance memory maintenance and confirming the results described in chapter 8.

Fig. 9.1. Experimental design of the experiments in this chapter.

The goal of this set of experiments was to test three things:

A, Memory performance after a short (30 min) and a long (24 h) delay (Fig. 9.2).

B, Effect of novel exploration on memory performance (Fig. 9.2).

C & D, Protein synthesis dependency of the effect of the exploration (Fig. 9.3).

E, F & G, Is performance in this task NMDAR dependent? And D1D5R dependent? Can the exploration rescue the effect of D1/D5 block? (Fig. 9.4).

H & I, Is the effect of the exploration D1D5R dependent? (Fig. 9.5).

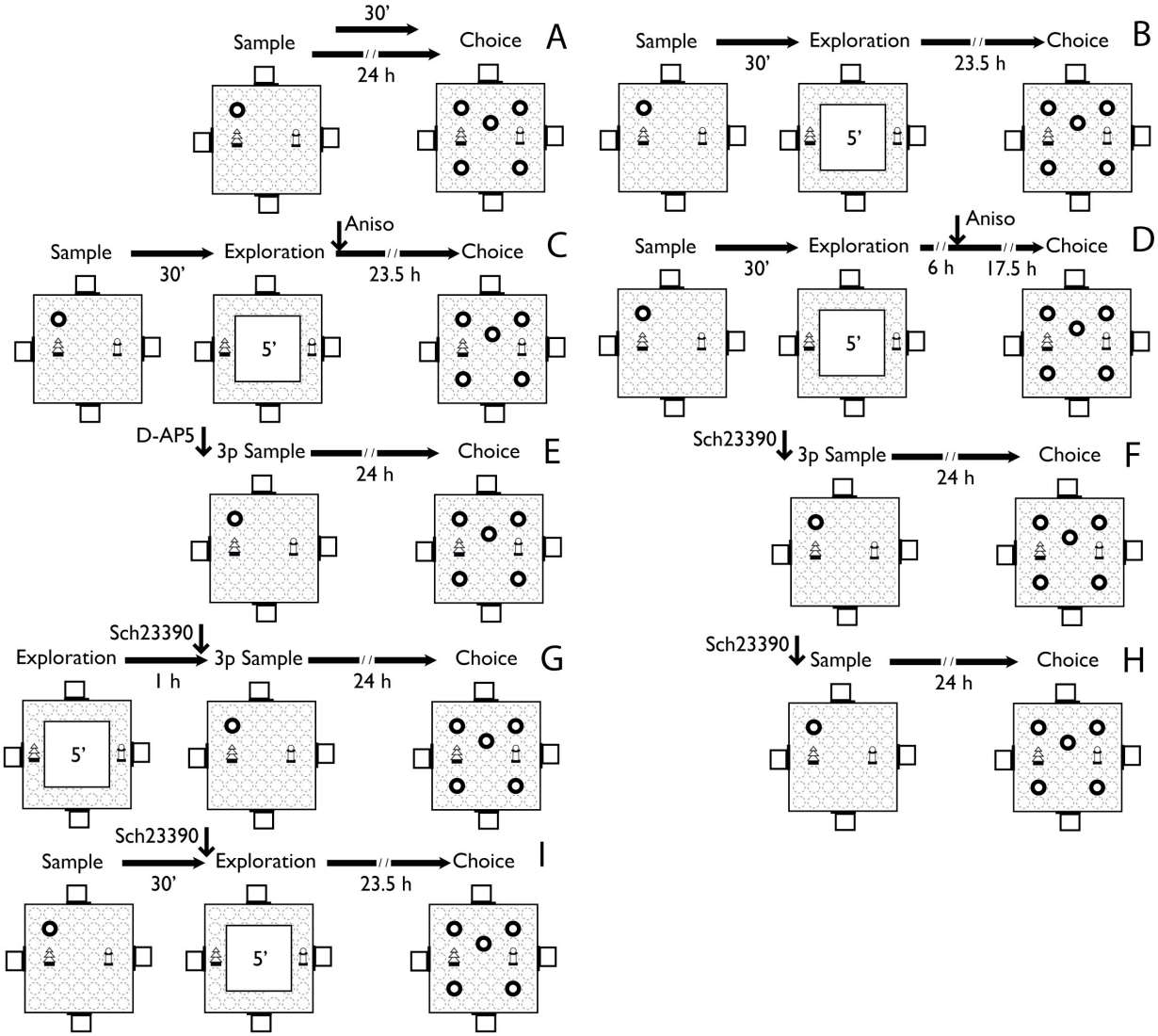
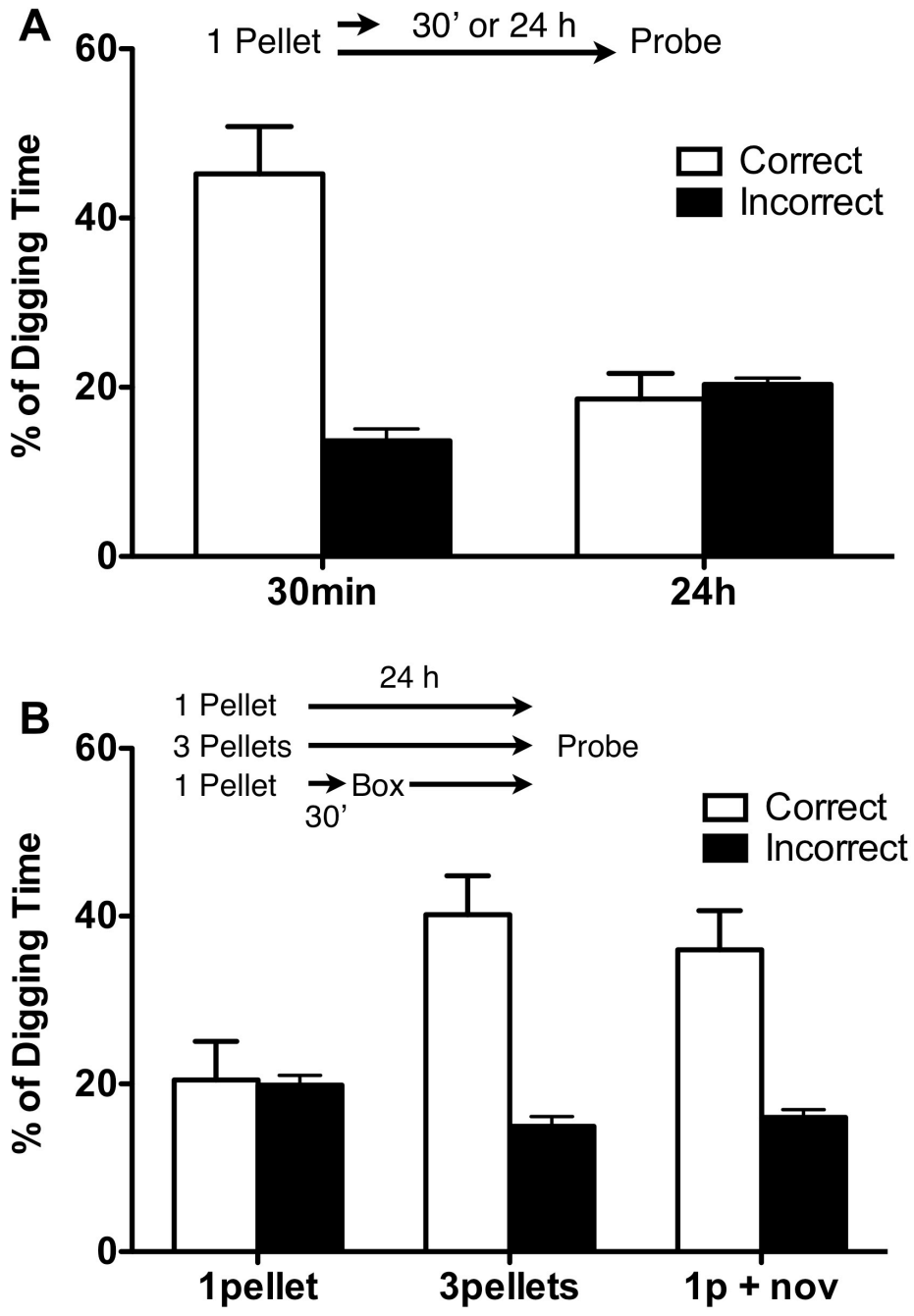

A
Sample
30'
24 h
Choice
B
Sample
30'
Exploration
23.5 h
Choice
5'
C
Sample
30'
Exploration
Aniso
23.5 h
Choice
5'
D
Sample
30'
Exploration
Aniso
6 h
17.5 h
Choice
5'
E
D-AP5
3p Sample
24 h
Choice
F
Sch23390
3p Sample
24 h
Choice
G
Sch23390
Exploration
1 h
3p Sample
24 h
Choice
5'
H
Sch23390
Sample
24 h
Choice
I
Sch23390
Sample
30'
Exploration
23.5 h
Choice
5'

Fig. 9.2. 'Weak' encoding produces a short-lasting memory that can be enhanced with the exploration of a novel environment.

A, Spatial memory of 1-pellet encoding lasted for 30 min (correct > wrong digging, t = 6.8, p < 0.001), but not for 24 h (no preference between correct and incorrect wells, t = 0.37, p > 0.5). The performance after 30 min was better than after 24 h (Bonferroni t = 5.73, p < 0.001) (n = 16).

B, When tested after 24 h, 1-pellet encoding produces no preference for the correct well (t = 0.13, p > 0.05) but there is memory when the exploration of a novel box is coupled to the 1 pellet encoding. (correct > wrong digging, t = 4.2, p < 0.001). When comparing conditions after 24 h, 1 pellet memory is weaker than 3 pellet (t = 4.14, p < 0.001); novelty enhances the memory after 1 pellet encoding (t = 3.26, p < 0.01) to an extent that makes the memory performance indistinguishable from that of a 3 pellet encoding (t = 0.88, p > 0.05). Data is presented in mean +/– s.e.m and analysed with t-tests with Bonferroni correction for multiple comparisons (n = 16).



9.3.2 Memory enhancement depends on protein synthesis

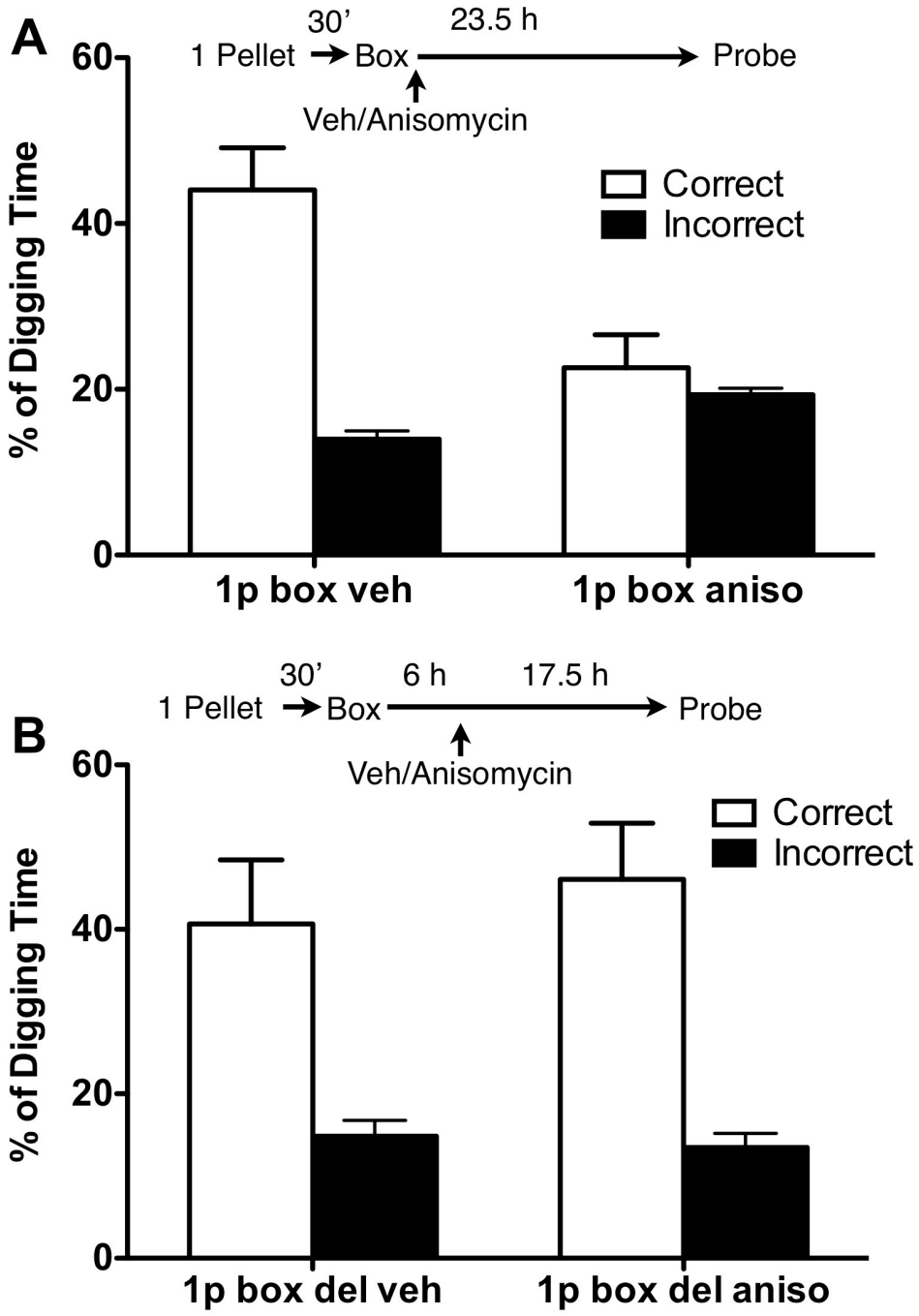
To support the hypothesis that the enhancement of memory is analogous to synaptic tagging, we next asked whether protein synthesis and D1/D5R activation in

the hippocampus, which is widely shown to be necessary for STC (Frey and Morris, 1997; O'Carroll and Morris, 2004) is also critical for this 'behavioural tagging'. To this end, we implanted cannula in the rat hippocampus to allow for local drug infusions (described in chapter 2). The rats recovered from the surgery and retrained to match pre-surgery performance. They were then given 1-pellet encoding followed by novel box exploration. Immediately or 6 hours after the encoding the animals received intra-hippocampus infusions of the protein synthesis inhibitor anisomycin (Fig. 9.3). The results showed that protein synthesis inhibition immediately after novelty blocks the novelty impact on the memory (Fig. 9.3A). A delayed protein synthesis inhibition, however, did not impair the memory (Fig. 9.3B). This suggests that novelty engages protein synthesis in the hippocampus and this is critical to enhance the memory maintenance. The critical time window for this to happen is within 6 hours of novelty exploration.

Fig. 9.3. Hippocampal protein synthesis is required for novelty enhancing memory maintenance.

A, (Top) Hippocampal protein synthesis is required for novelty-enhanced memory persistence. Exploration in a novel box 30 min after the encoding enhanced the 1-pellet memory persistence (Veh, correct > wrong digging, $t = 6.46$, $p < 0.001$), which was impaired by hippocampal anisomycin infusion immediately after the box exploration (similar correct and wrong digging, $t = 0.69$, $p > 0.5$). The performance between the two conditions was also significantly different ($t = 6.61$, $p < 0.001$) ($n = 11$).

B, On the other hand, a 6 h - delayed anisomycin infusion did not impair the memory (correct > wrong digging in Veh, $t = 3.43$, $p < 0.05$ while in the delayed anisomycin group, $t = 4.33$, $p < 0.01$). There is no difference between the anisomycin and the vehicle condition ($t = 0.72$, $p > 0.05$). Data is presented in mean +/– s.e.m and analysed with T-tests with Bonferroni correction for multiple comparisons ($n = 11$).


A
30'
23.5 h
1 Pellet
Box
Probe
Veh/Anisomycin
Correct
Incorrect
% of Digging Time
60
40
20
0
1p box veh
1p box aniso
B
30'
6 h
17.5 h
1 Pellet
Box
Probe
Veh/Anisomycin
Correct
Incorrect
% of Digging Time
60
40
20
0
1p box del veh
1p box del aniso

9.3.3 The encoding of the strong memory requires NMDAR and D1/D5R activation

To assess whether the encoding of the spatial memory itself requires hippocampal D1/5R in the event arena task as in the water maze task (O'Carroll et al., 2006) we trained the rats to encode the correct location with 3 visits (3 pellets trial, see methods chapter 2) followed by a probe test at a 24-hour delay. This stronger encoding is crucial as it reveals good spatial memory at the longest interval tested (24 h) while still requiring NMDAR activation in the hippocampus (Fig. 9.4A), similar to a weaker encoding scenario.

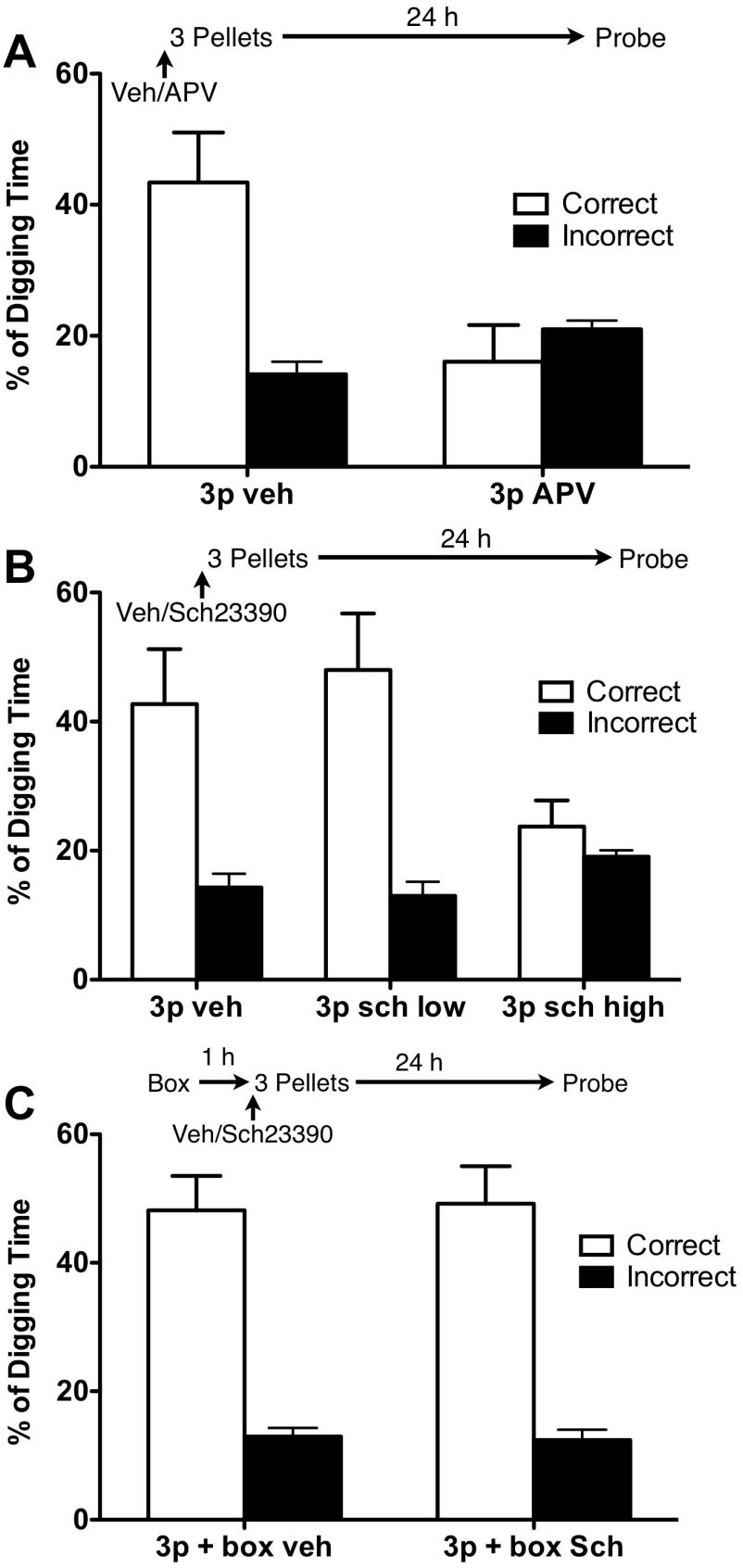
We then infused Sch23390 (low or high dose) or vehicle into the hippocampus before 3-pellet encoding. The results showed that a lower dose that was sufficient to impair novelty impact on the spatial memory was insufficient to impair the memory retention for 24 hours (Fig. 9.4B). It is possible that 3-pellet encoding phase triggers stronger dopamine release, which then requires a stronger D1/5r blockade to detect memory impairment. Indeed, a higher dose of Sch23390 impaired the memory retention of 3-pellet encoding (Fig. 9.4B). Remarkably, novelty exploration, given before 3-pellet encoding, could completely rescue the impairment caused by Sch23390 (Fig. 9.4C).

Fig. 9.4. The encoding of the strong memory requires NMDAR and D1/D5R activation.

A, NMDAR antagonist, AP5, in the hippocampus impaired the memory retention of 3 pellets (Veh, correct>wrong digging, $t = 4.23$, $p<0.01$, AP5, $t = 0.72$, $p > 0.05$). The difference between conditions was also significant ($t = 3.98$, $p < 0.001$) ($n = 11$).

B, The 24h memory retention of 3 pellets was impaired by a higher dose of Sch23390 (3.3 μg/μl/hemisphere, correct and wrong were indistinguishable, $t = 0.62$, $p > 0.05$) but not by a lower dose of Sch23390 (1μg/μl/hemisphere) or vehicle (Sch(low), correct>wrong digging, $t = 4.58$, $p < 0.001$; and Veh, $t = 3.72$, $p < 0.01$). While the vehicle condition was indistinguishable from the low dose of Sch23390 condition ($t = 0.69$, $p > 0.05$), the performance after the infusion of high dose of Sch23390 was lower than vehicle ($t = 2.48$, $p < 0.05$) and the low Sch23390 ($t = 3.17$, $p < 0.01$) ($n = 11$).

C, Exploration in a novel box 1h before the encoding completely rescued the memory impairment by the higher dose of Sch23390 (correct vs. incorrect digging; $t = 6.35$, $p < 0.001$), as now, after the exploration of the novel box, the performance in pre-encoding Sch23390 infusion group was similar to vehicle infusion group ($t = 0.18$, $p > 0.05$). Data is presented in mean +/– s.e.m and analysed with T-tests with Bonferroni correction for multiple comparisons ($n = 11$).


A
3 Pellets
24 h
Probe
Veh/APV
% of Digging Time
60
40
20
0
Correct
Incorrect
3p veh
3p APV
B
3 Pellets
24 h
Probe
Veh/Sch23390
% of Digging Time
60
40
20
0
Correct
Incorrect
3p veh
3p sch low
3p sch high
C
Box
1 h
3 Pellets
24 h
Probe
Veh/Sch23390
% of Digging Time
60
40
20
0
Correct
Incorrect
3p + box veh
3p + box Sch

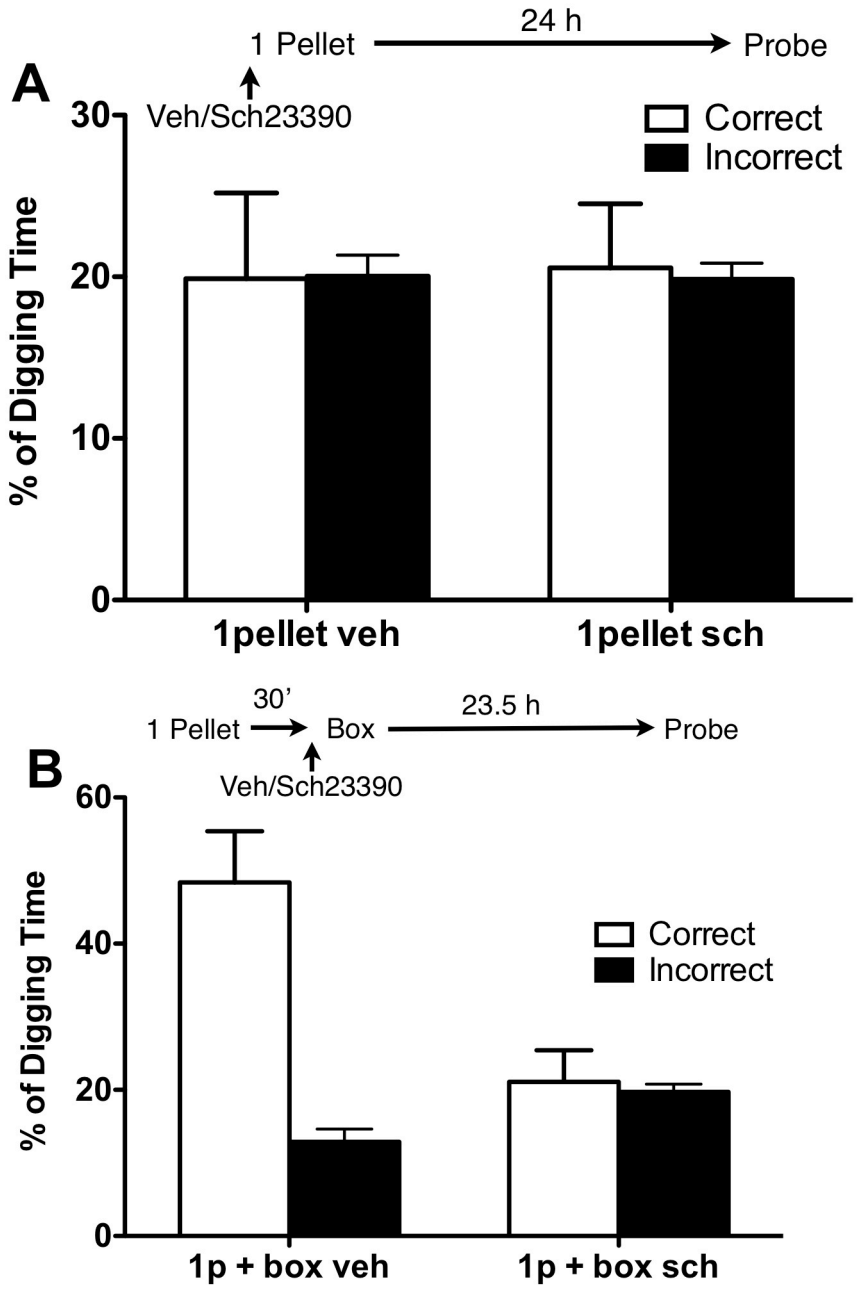
9.3.4 D1/D5 receptor activation is necessary for the effect of novel exploration

We then asked whether the novelty exploration effect is mediated by D1/5 receptors in the hippocampus. Rats encoded the location of 1-pellet well and received intra-hippocampus infusions of D1/5R antagonist, Sch23390, followed by novelty exploration or by returning to the home cage (Fig. 9.5A). The results showed that rats dug randomly at all wells during probe test if encoding of 1 pellet was not followed by novelty exploration (Fig. 9.5B left). When the encoding of 1 pellet was followed by novelty exploration, the memory was maintained for 24 hours. This effect was impaired by the D1/5R antagonist before novelty (Fig. 9.5B). This suggests for novelty exploration to enhance memory maintenance it requires the integrity of dopamine transmission through D1/5r in the hippocampus.

Fig. 9.5. Dopamine and the novel box exploration.

A, Hippocampal infusions of vehicle or D1/5R antagonist, Sch23390 (1μg/μl/hemisphere), after encoding did not enhance the 1-pellet memory maintenance (t = 0.14, p > 0.05) (n = 11).

B, On the other hand, exploration in a novel box 30 min after the encoding produced significant performance after 24 h (Veh, correct>wrong digging, t = 5.91, p < 0.001), which was impaired by hippocampal Sch23390 infusion before the box exploration (Sch, both correct and wrong digging at around chance level, 20%, t = 0.23, p = 0.81). The difference between conditions was also significant (t = 4.55, p < 0.001). Data is presented in mean +/– s.e.m and analysed with T-tests with Bonferroni correction for multiple comparisons (n = 11).


A
1 Pellet
24 h
Probe
Veh/Sch23390
Correct
Incorrect
% of Digging Time
30
20
10
0
1pellet veh
1pellet sch
B
30'
1 Pellet
Box
23.5 h
Probe
Veh/Sch23390
% of Digging Time
60
40
20
0
Correct
Incorrect
1p + box veh
1p + box sch

9.3.5 Familiarization to the novel box, time and location of the exploration

At the end of the experiment we conducted some controls on the mechanisms behind the 'behavioural tagging' studied in this chapter. With the animals well trained after months involved in the same task, we tested whether the animals were using an allocentric strategy to solve the task. We confirmed that even after switching the start location from one position during the encoding trial to another during the retrieval phase, the rats were performing above chance and at the same level than with a constant start box (Supplemental Fig. S4).

We also tested the animals' reliance on the cues around the experimental room by removing the two intramaze cues during retrieval. Also in this case, their memory after 1 pellet encoding and a delay of 30 min was above chance and the same as controls (Supplemental Fig. S4).

We were also interested in whether the animals could maintain some form of memory of the novel box, and particularly, of the substrate that made the box 'new' at every exposure. Tracking software measured the path length of the rat as it explored the novel box for the typical 5 min exploration and also after a 24 h delay. There was a reduced path length on the second exploration period suggesting less interest and maybe consequently a memory of the first exploration (Supplemental Fig. S6).

Finally, we tested the timing requirements for the effect of the exploration (Supplemental Fig. S7A). The exploration of the novel box improved memory retention when presented 30 min after the encoding but not when the exploration was delayed 6 h.

One additional variable pending from the preliminary experiments described in chapter 8 concerns the requirement for the exploration to take place in the same environment as the encoding of the memory. For the behavioural effect described in this chapter to fit into a behavioural correlate of the STC hypothesis, the exploration should affect the same cells encoding for the location of the correct location. To maximize that probability, emphasis was put on the exploration to take place in the same context as the one-trial match to place task, in the hope of obtaining an overlap

between cells encoding the novel box and those encoding the location of the correct well. To test this, a 1 pellet memory was followed by the exploration of a novel box in an identical event arena located in a different room (i.e. with different cues). Also in this case, there was a rescue in the decay of the memory such that the performance 24 h after the encoding was above chance (Supplemental Fig. S7B).

In the experiments described in this chapter the animals were never familiarized with any of the novel box configurations. The question remained as to whether the effect of the novel box could also be obtained with the exploration of the event arena for 5 min. When the novel box exploration was replaced by the exploration of the event arena there was no memory left 24 h after the encoding of the weak 1 pellet version of the task (Supplemental Fig. S7B).

9.4 Discussion

This chapter describes a dopamine and protein-synthesis dependent behavioural manipulation capable of enhancing the maintenance of a NMDAR and dopamine-dependent spatial memory. The design of these experiments mimics the electrophysiology of hippocampal synaptic plasticity and the results follow the mechanisms described by the STC hypothesis.

Briefly, rats can be implanted with a weak (< 24 h) memory for the location of food reward by visiting the location once (Fig 9.2). This type of spatial memory can last 24 h if the location is visited three times instead. As seen in chapter 9, the exploration of a novel environment after the encoding of the weak memory allows this memory to last for at least 24 h.

The synthesis of PRPs could be the mechanism of action of the novel box exploration as its effects are blocked by anisomycin (Fig 9.3). This effect is conditional to the time of infusion of the drug since delayed applications of anisomycin had no effect.

Importantly, the strong memory encoded by three visits to the rewarded location needs functional NMDARs in the dorsal hippocampus at the time of encoding (Fig 9.4A). Besides showing the hippocampal dependency of this task, this type of experiments (Morris et al., 1986) goes one step further and suggests a similarity between the behavioural mechanisms of memory formation in this task and the mechanisms responsible for changes in synaptic efficacy at the synapse level described in chapter 3.

The encoding of the spatial location in the one-trial match to place task in the event arena also requires the activation of dopaminergic D1/D5R at the time of encoding (Fig 9.4B). This requirement matches electrophysiological data on synaptic plasticity showing the impairment in LTP maintenance if D1/D5 receptors are blocked at the time of induction (Frey et al., 1990; Frey et al., 1991; Otmakhova and Lisman, 1996; O'Carroll and Morris, 2004).

There is other behavioural data on the role of dopamine in spatial memory showing the need for dopaminergic input (Gasbarri et al., 1996) or D1/D5R activation in order to solve one trial memory tasks in the watermaze (O'Carroll et al., 2006).

D1/D5Rs bind and stimulate adenylyl cyclase (Kimura et al., 1995) increasing the concentration of cAMP and activating PKA, which is necessary to engage the synthesis of new PRPs (Abel et al., 1997; Nayak et al., 1998). If the novel exploration enhances the weak memory by allowing the synthesis of PRPs and if what the Schering compound blocks in the 3-pellet version of the task is precisely the pathway leading to new PRPs, the pairing of the novel exploration with the D1/D5R-blocked 3-pellet encoding should rescue the memory. Indeed, the memory impairment seen with D1/D5R blockage during the encoding of the strong memory can be rescued by the novel exploration (Fig 9.4C). This also fits the same mechanisms of action as those described previously by Li and others (Li et al., 2003; Moncada and Viola, 2007),

Stronger evidence for the role of dopamine can be seen when working with the weak encoding event (1 pellet). First, there is no detectable effect of Schering 23390 application during the encoding (Fig 9.5). However, if D1/D5R are blocked during the exploration of the novel box, the rescue of the weak memory after 24 h is lost.

To summarize, our findings suggests that at the behaviour level, 'tagging and capture' phenomenon can be observed in spatial learning, similar to the findings at synaptic level. The implications of this finding as well as the potential uses of a behavioural task capable of translating the discoveries in synaptic plasticity into spatial memory are evaluated in the final chapter to this thesis.

Chapter 10: Behavioural tagging and cold water stress

10.1 Introduction

The aim of this study was to test a behavioural prediction of the 'synaptic tagging and capture' hypothesis (Frey and Morris, 1998a). As introduced in chapter 1, a behavioural correlate of the STC hypothesis should be capable of turning a memory that ordinarily decays over a short period of time into a longer lasting memory by means of a behavioural intervention that triggers protein synthesis around the time of memory encoding. Chapters 8 and 9 have demonstrated the possibility of obtaining such behavioural correlate by using the exploration of a novel environment. Could a similar result be obtained in the watermaze by using cold water stress?

10.1.1 Upregulation of PRPs by cold-water stress

We hypothesized that a cold water experience could bring plasticity proteins into play since cold stress elevates corticosterone levels but does not enhance or impair LTP in the DG (Bramham et al., 1998). Even better, swimming in colder water (19 degrees C) improved memory with respect to warm water (25 degrees) in the watermaze (Akirav et al., 2004). From these observations, we predicted that acute cold stress (dunking of the animal for less than 1 min in a cold water bucket) could produce the PRPs necessary to maintain a weak memory (platform location in the watermaze).

However, Diamond and Woodson, 2004 show that watermaze platform memory is clearly impaired after 30 min if combined with a stressful event (electric shock) (Diamond et al., 2004). They claim that the impairment is due to the new stressful memory being encoded and disturbing the distribution of synaptic weights that would encode the platform location. This could be similar to our cold swim after encoding phase and invalidate our prediction.

The electrophysiology shows that behavioural stress facilitates the induction of long-term depression (LTD) in hippocampus (Xu et al., 1997; Chaouloff et al., 2007). It does so by acting on NR2B containing NMDARs and without using NR2A or synaptic NMDARs (Yang et al., 2005). This suggests that LTD observed in slices from stressed animals is triggered primarily by extrasynaptic NMDAR activation. Yang also finds that the facilitation of LTD in stressed rats is due to the blockade of glutamate uptake. The risk of using stress as a behavioural manipulation can be summarized as follows: Behavioural stress blocks glutamate uptake, therefore enhancing LTD, which can be excitotoxic in nature. The consequent modifications in synaptic weights would interfere with the correct encoding or maintenance of relevant memories. Despite the fact that stress could induce synaptic depression, the cold water manipulation was still worth using as a PRP-inducing protocol as STC experiments have revealed the phenomenon of cross-tagging in which regardless of whether the upregulation of PRPs is triggered by the induction of potentiation or depression mechanism, weakly tagged synapses are capable of capturing the PRPs (cross-tagging (Sajikumar and Frey, 2004a)). Also, it is worth remembering that LTD could serve as a mechanism for memory storage as well as LTP (Bear and Abraham, 1996; Kemp and Manahan-Vaughan, 2004). Because of this potential role of LTD in the encoding of memories, these electrophysiological results support the prediction of stress engaging plasticity mechanisms. Cold-water stress was used in this experiment in an attempt to upregulate the PRPs necessary to stabilize a weak memory thereby lengthening its persistence.

10.2 Methods

10 male Lister Hooded rats were trained in the delayed-match-to-place (DMP) task in the watermaze as described previously (O'Carroll et al., 2006). All experiments were counterbalanced and in a within subjects design (see chapter 2 for general methodological description). The stressful event consisted of a 1 min cold-water swim on a deep sink in a separate room to the watermaze.

During probe trials, performance was measured both as the latency to reach the location of the platform (kept deep below the surface out of reach of the rat) and as the percentage of the 60 s exploration time that the rat spent swimming within 30 cm of the platform location. This probe test with the lowered platform was carried out on trial number 2 of a series of 4 trials (see chapter 2 for detailed methodology of the DMP task).

After 4 days of regular training we tested the rats performance on the DMP task by a massed encoding protocol consisting of three swim trials with 30 s intertrial interval (ITI). This performance was measured 1 min and 6 h after encoding and served as a measure of maximal performance in the task before the behavioural manipulation was introduced.

On days 9 to 12 the animals were allowed to swim to the platform location for the day. 20 min before the encoding, some animals experienced a 1 min cold water swim or rested in their cages. Their performance was tested 1 min or 6 h after the placement.

10.3 Results

10.3.1 Effect of cold swim stress given 20 min before a swim trial on memory performance 1 min and 6 hours after encoding.

Latency to reach platform

As explained in the methods above, the interesting trial in the DMP task is trial number 2, after the encoding of the new location of the day and after the delay introduced by the experimenter. When compared to the good memory produced by the 3-swim ('strong') encoding protocol at the end of training, the 'weak' encoding protocol (1 swim to the platform location) allowed the animals to successfully swim to the platform with a short latency if 1 min was allowed between encoding and retrieval, but their performance was poor if instead of 1 min, 6 h were allowed to pass (Bonferroni comparing strong vs. weak encoding performance in the control condition 6 h delay; $t = 4.63$, $p < 0.001$ Fig. 10.1).

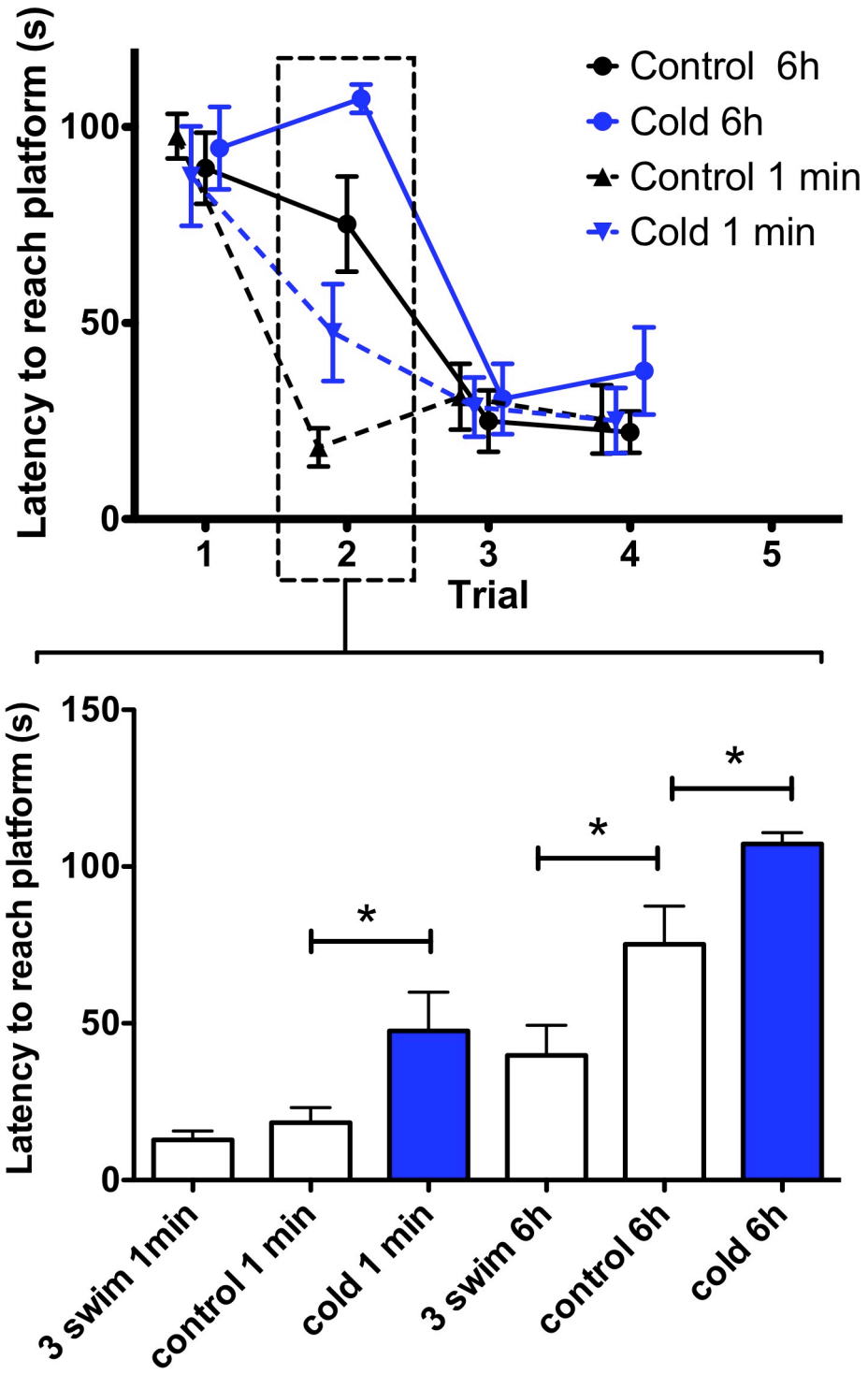
The behavioural manipulation (cold swim stress) impaired the overall latencies of the rats (2-way ANOVA $F_{1,28} = 10.96$, $p < 0.01$) with a significant impairing effect at the long retention time of 6 h (Bonferroni $t = 2.47$, $p < 0.05$) and a trend for impairment also at the short time delay of 1 min (Bonferroni $t = 2.26$, $p > 0.05$) (Fig 10.1). The fact that the memory is nearly worse than controls after 1 min suggests that there is a defective encoding of the information and predicts the memory deficiency observed after 6 hours.

The latency measurement is a standard tool to measure performance in this task but as with most measures, there is the risk of not seeing an effect due to ceiling or floor effects. In this experiments, the animals may be so good after a 1 min delay that they show a ceiling effect that occludes the effect of the cold swim. Fortunately, there are other measures (i.e. e. savings and percentage time around the platform) that can be used to assess the effect of this behavioural manipulation in memory performance (see chapter 2, next section and discussion).

Figure 10.1. Latencies to reach the correct platform location.

A, Trial by trial performance (latency scores of 10 animals). The treatment (Cold swim for 1 min 20 min before the encoding trial (trial 1)) impairs memory performance on trial 2 (Two-Way ANOVA of trial 2 latencies, $F_{1,18} = 10.96$, $p < 0.01$). The fact that the memory is already worse in the cold-swim condition already after 1 min (Bonferroni $t = 2.26$, $p > 0.05$)) suggests that there is a defective encoding of the information and predicts the memory deficiency observed after 6 hours (Bonferroni $t = 2.47$, $p < 0.05$).

B, Bar graph comparing the latencies during trial 2 of the 3-swim strong-encoding protocol used at the end of training and the two conditions of this experiment (1 swim with or without cold-stress treatment). There are overall differences between the 6 conditions in this graph (One-way ANOVA $F_{5,45} = 20.01$, $p < 0.001$) and in particular, there is evidence for the 1 swim protocol inducing a decaying memory that weakens after 6 h when compared to the performance of the 3-swim encoding condition (3-swim protocol performance at 6h vs. 1-swim protocol, $t = 3.13$, $p < 0.01$). Also, the cold-swim treatment impairs memory both after 1 min (Bonferroni $t = 2.59$, $p < 0.05$) and after 6 h (Bonferroni $t = 2.83$, $p < 0.05$).


Latency to reach platform (s)
100
50
0
1
2
3
4
5
Trial
Control 6h
Cold 6h
Control 1 min
Cold 1 min
150
100
50
0
*
*
*
3 swim 1min
control 1 min
cold 1 min
3 swim 6h
control 6h
cold 6h

Savings in time between trial 1 and 2

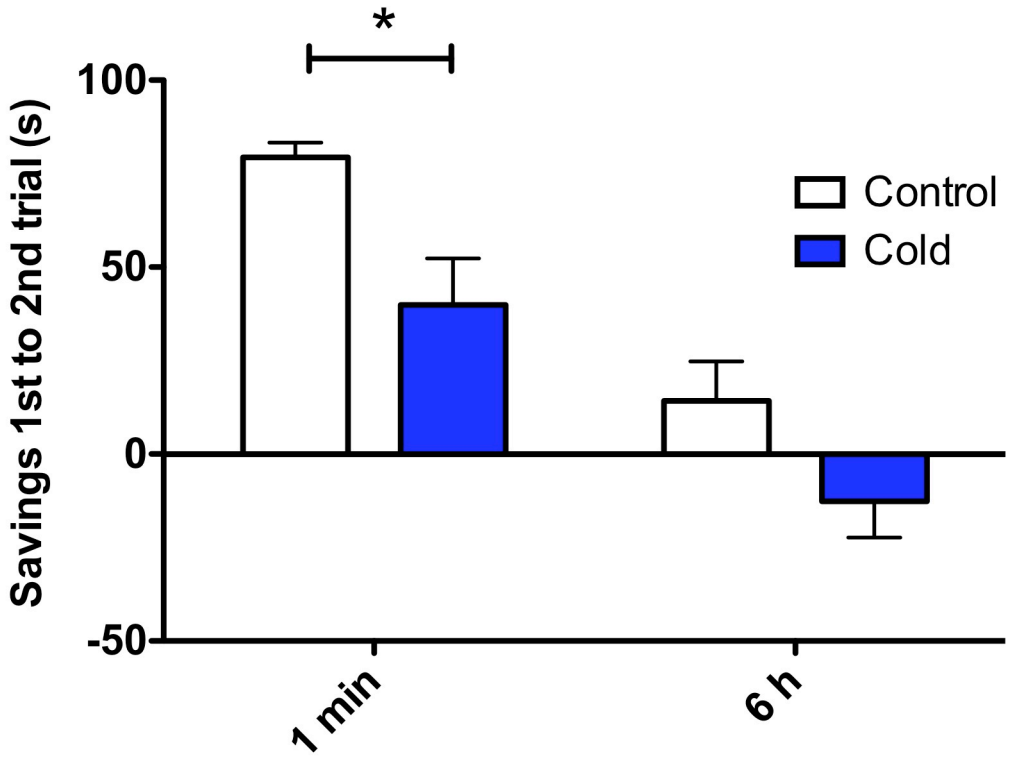
Another way to look at the performance on trial 2 of this task takes into account the performance on the first trial. By subtracting the time that it takes to find the platform on trial 2 (latency) from the latency to find the platform on trial 1, one can measure the saving in search time that an accurate memory of the platform location allowed on the second trial. The larger the saving is, the better the performance in trial 2 with respect to trial 1 (Fig. 10.2). The comparison of savings also reveals an overall negative effect of the cold swim manipulation (2-way ANOVA $F_{1,18} = 12.59$, $p < 0.01$). Interestingly, the measurement of savings shows a significantly negative effect of the cold swim treatment at the short delay (1 min) (Bonferroni $t = 2.89$, $p < 0.05$) but this way of measuring performance fails to find a significant effect of the cold treatmen at the long delay (6 h) (Bonferroni $t = 1.97$, $p > 0.05$).

The latency and the savings measurements as used in this chapter are a good example of ceiling and floor problems in behavioural tasks (see discussion).

Also in the savings measure, the memory after cold swim treatment is already worse after 1 min suggests that there is a defective encoding of the information and predicts the memory deficiency observed after 6 h.

Figure 10.2. Savings to reach the correct platform location after 1 swim trial (trial 1).

The saving measure is obtained by substracting the latency to find the platform on trial 2 from the original latency in trial 1. On this measure, the treatment (Cold swim for 1 min 20 min before the encoding trial (trial 1)) impairs memory performance both after 1 min and after 6 hours (Two-Way ANOVA of trial 2 latencies, $F_{1,18} = 12.59$, $p < 0.01$). The savings are significantly lower even after just 1 min (Bonferroni $t = 2.89$, $p < 0.05$)) although these savings measure fails to find a difference after 6 h (Bonferroni $t = 1.96$, $p > 0.05$).



10.3.2 Cold swim does not alter the percentage of time spent around the platform

With the use of the Atlantis platform (described in methods chapter 2), there is one more variable that is informative as to the performance in the DMP memory task: the percentage of the trial time spent around the platform before it is raised and the animal finds it. This measurement is based on the selection of an area around the platform (i.e. 30 cm diameter circular area) and benefiting from the analysis built into the tracking software to calculate what percentage of the total time of the trial did the animal spend swimming within the specified area.

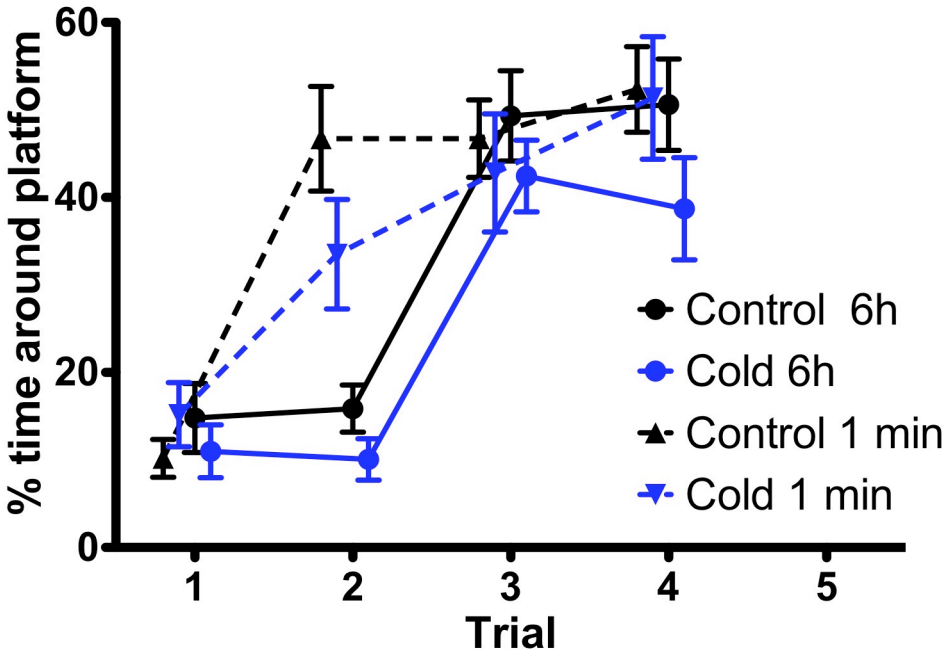
As shown in Figure 10.3, the treatment with the cold swim before the encoding phase fails to improve the performance of the rats on the retrieval trial (second trial of the day) (2-way ANOVA $F_{1,18} = 3.58$, $p > 0.05$). There was no difference in performance when comparing conditions that involved encoding by 1 swim to the platform and those trials that before this encoding, had the animals exposed to the 1 min swim in cold water. This happened both at the short delay of 1 min (Bonferroni $t = 1.99$, $p > 0.05$) as well as at the longer delay 6h (Bonferroni $t = 0.88$, $p > 0.05$).

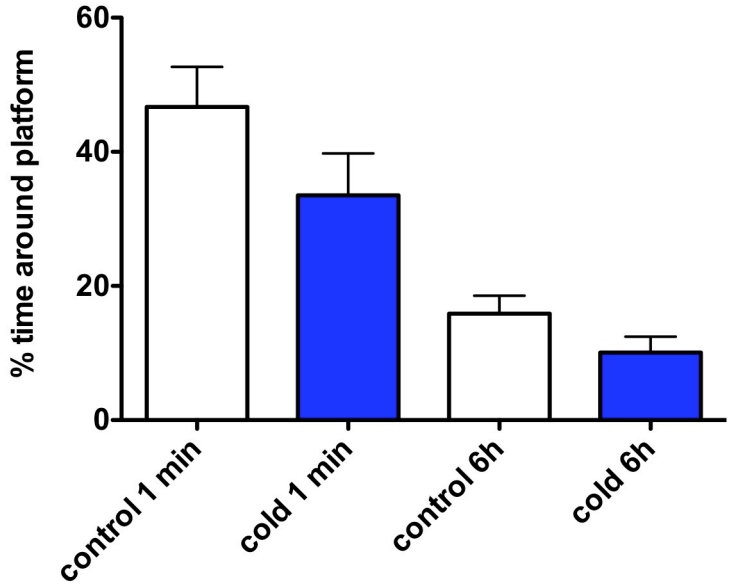
To summarize, the stress used in this experiments had the opposite effect of what was expected.

Fig 10.3. Memory performance as percentage of time spent around the correct location.

A, Percentage of time spent around 30 cm of the platform location during a probe trial for the 4 trials of a session. While all animals perform equally well during trial 1 (encoding trial) there performance in trial 2 is worsened by the time delay between trials 1 and 2 (Two-way ANOVA $F_{1,18} = 39.62$, $p < 0.001$). However, there is no effect of the cold treatment (Two-way ANOVA $F_{1,18} = 3.58$, $p > 0.05$).

B, A cold swim for 1 min 20 min before the encoding trial (trial 1) does not enhance memory performance as seen in trial 2 data.


% time around platform
60
40
20
0
1
2
3
4
5
Trial
Control 6h
Cold 6h
Control 1 min
Cold 1 min


% time around platform
60
40
20
0
control 1 min
cold 1 min
control 6h
cold 6h

10.4 Discussion

These experiments describe an attempt to find a behavioural correlate of synaptic tagging by testing whether the maintenance of weak spatial memory could be lengthened by providing a stimulus capable of making available the PRPs necessary to stabilize the memory to the cells encoding the memory.

The strength and persistence of the memory for the spatial location of an escape platform in the Morris watermaze can be controlled by manipulating the number of encoding trials (i.e. number of times that the rat is given the opportunity to swim and find the hidden platform). Therefore, we used a weak encoding protocol (i.e. one swim to the platform) capable of inducing a memory that could be detected after a short time interval (1 min) but was very weak after a longer delay (6 h) (Figure 10.1).

The question could then be asked as to whether a behavioural manipulation would be capable of enhancing the memory. The ultimate goal is to find a mechanism whereby IEGs can be upregulated in the cells participating in the engram of the memory (Martin et al., 2000b). The first step, as explained in the introduction to this chapter, would be to benefit from the known effects of mild stress in the learning of a spatial location in the watermaze (Sandi et al., 1997). Rats were allowed to experience 1 min swim in a sink filled with cold water (13 º C) 20 min prior to the encoding session (1 swim to the platform). In a within subject design, the effect of this cold water stress could be assessed both at the 1 min and at the 6 h time interval.

All measurements of performance (the latency to reach the location, the savings from trial 1 to 2, and the percentage of time spent close to it) were negatively affected by the cold-water stress (Figure 10.1-3). Interestingly, the effect was seen both at the long retention interval (6 h) as well as in the short delay (1 min test). This suggests that the cold-water stress had a negative effect on the strength of the encoding. Overall, there is failure of this protocol to enhance the retention of this place memory.

10.4.1 The variable effects of stress on memory

The negative effect of stress on memory described in this chapter may be due to the bell-shaped curve of effects of stress on memory performance (Akirav et al.,

2004). Stressful events may be unsuitable memory modulators when searching for a behavioural correlate of STC due to the risk of overstressing and the lack of control over the subjective experience of stress by the individual animals. When studying the effects of stress and fear in a hippocampal task there is also the additional complication of multiple brain structures combining their actions. Fitting the data shown in this chapter, tail-shock stress impaired LTP in the hippocampus and it also impaired memory performance in the watermaze (Kim et al., 2005). Interestingly, the inactivation of the amygdala (with GABAa agonist muscimol) before but not after stress rescued the memory and prevented the LTP impairment. At the molecular level, stress can act through two types of corticosteroid receptors: the high-affinity mineralocorticoid receptor (MR) that is most densely localized in hippocampal and septal neurons or the glucocorticoid receptor (GR) that is ubiquitously distributed in the brain, including neurons in the hippocampus, hypothalamus, glial cells, and pituitary cells. The MRs are activated at low corticosterone (CORT) concentrations while high concentrations may activate GRs. Indeed, an inverted U-shaped curved has been seen to describe the effects of corticosterone in a form of hippocampal plasticity (Diamond et al., 1992) and the characteristics of these two receptors could account for two stress systems with different roles in survival (de Kloet, 2003). Fitting these models, LTP in DG is impaired by 15 min swim (stress) and this is reversed by GR but not by MR antagonist suggesting that MRs are required for LTP while GRs can block it (Avital et al., 2006). The effect of water stress on LTP and LTD in the CA1 region of the HPC have recently been reported to differentiate between dorsal and ventral hippocampus (Maggio and Segal, 2009). This complex picture behind the effects of stress could account for the impairment of the cold swim as a behavioural manipulation. More importantly, the complexity of the actions of stressful events directed this thesis' search for a behavioural correlate of STC away from stress and towards an alternative mechanism that could engage the synthesis of PRPs (see chapters 9 and 10).

10.4.2 Towards a better behavioural manipulation capable of revealing the phenomenon of behavioural tagging

As it was described in chapter 8, dopamine release in the dorsal hippocampus has a necessary role in engaging the molecular machinery leading to the availability of PRPs. This is also the case in the electrophysiology of LTP and STC where D1/D5R antagonists do not allow the maintenance of LTP (Frey et al., 1990; Frey et al., 1991; O'Carroll et al., 2006). Behaviourally, the facilitation of LTP by spatial exploration is dependent on dopamine (Li et al., 2003). Taking this into account, we know that midbrain dopamine neurons are activated by appetitive but not aversive stimuli (Mirenowicz and Schultz, 1996) and this could also explain why the aversive cold-swim experience did not facilitate the maintenance of the spatial memory tested in this chapter.

A theoretical assumption for the successful translation of STC into behaviour may be the requirement for an overlap in the identity of the cells activated by the encoding event and those where PRPs are made available by the modulatory experience (Vazdarjanova and Guzowski, 2004). Assuming that the targets of the modulatory event have to be the place cells encoding the spatial location, the success of the modulatory event may be helped by experiencing it in the same environment where the rats encode the place memory. The nature of the watermaze as a task limits the ways in which to present a PRP-modulating event within the watermaze. This, together with the inherent stress of the swimming task, led to the pursuit of a behavioural correlate of the STC hypothesis in the ‘event arena’, as described in chapters 8 and 9.

Chapter 11 General Discussion

The experiments described in this thesis have established dissociations in CaM kinase signalling pathways with respect to protein synthesis-dependent L-LTP, and shown that a short-term memory can be transformed into a long-term memory by a task-independent treatment. These findings and their implications have depended on the development of new experimental tools.

11.1 Developing the right tools to learn about the late phases of synaptic change.

The findings described in chapters 3 to 7 underscore the value of allowing newly prepared brain slices to stabilize for at least 4 h, with minimal test stimulation, before any tetanization protocol was applied. Biochemical data suggest that low frequencies of test stimulation interfere minimally with PRP availability (Fonseca et al., 2006a) and that it takes many hours (i.e. more than 4 h) for kinase activation levels to stabilize after slice preparation (Ho et al., 2004). With this in mind, we conducted our experiments at very low rates of test stimulation and allowed the slices to rest for at least 4 h before testing any drug or high frequency stimulation.

Together with the control of the temperature in the whole of the electrophysiology rig and the control of the humidity in the slice chamber (chapter 2), we managed to record for much longer periods, e.g. 10 h, than is typical in many LTP experiments (Sajikumar et al., 2005b). Recording for extended periods makes it desirable, and arguably essential, to have a third non-tetanized pathway against which to assess the magnitude of LTP long after tetanization. Our data provide various indications that the dynamic interactions set in train by tetanization continue for several hours. For example, in chapter 4, weak tetanization induces an LTP that does not decay to baseline until 4 to 6 h have elapsed. In chapter 6 the cross-over of the strong and weakly tetanized pathways takes place over a period from 2 to 6 h after tetanization, and the strongly tetanized pathway does not decay to baseline until 8 to 10 h have elapsed. The development of a stable slice preparation has been critical to dissecting the differential role of distinct CaM kinases with respect to synaptic tagging and capture (chapter 2).

11.2 Pharmacological dissection of molecular roles in synaptic plasticity

The experiments described in this thesis have identified dissociable roles specific for distinct CaM Kinase pathways in the maintenance of synaptic potentiation, and they provide new molecular insights on the requirement for synapse-specific and cell-wide actions during STC. Our findings emerge from our use of long time course 3-pathway protocols (i.e. 2 pathways tetanized in 'weak-before-strong' and 'strong-before-weak' modes, with a third non-tetanized control pathway), which could be readily combined with fast wash-in/wash-out pharmacological treatments (see methods section 2.1.1)

First, in chapters 3 to 5 we confirm previous observations (Frey and Morris, 1997; Fonseca et al., 2004), that E-LTP induced at one set of synapses can be rescued into L-LTP if, within a short time-window (Frey and Morris, 1998b), L-LTP is induced at another set of synapses in the same CA1 neuronal population. Second, in chapter 6 cell-biological data reveals differential concentration-response actions of the CaMKII inhibitor KN-93 for candidate synapse-specific and cell-wide mechanisms. A low concentration of KN-93 selectively interrupted a pathway-specific tagging process, but spared a cell-wide supply of PRPs, as that is analytically revealed by successful L-LTP after weak tetanization of a second independent pathway. Third, a higher concentration of KN-93 blocked L-LTP without 'tag' specificity. This result was probably not due to unspecific effects of KN-93 and builds upon our own 'strong before strong' experiments with KN-93 and earlier work with KN-62 (Sajikumar et al., 2007). Fourth, in chapter 7 we established that the CaMKK inhibitor STO-609 blocks L-LTP but does so by interrupting a cell-wide process that can be overcome by strong tetanization of a separate pathway.

11.3 The STC hypothesis revisited

Central to this thesis is the rescue of E-LTP into L-LTP through the interaction between two inputs of different strength onto the same cell - a form of heterosynaptic metaplasticity that can be best explained by the synaptic tagging and capture hypothesis (Frey and Morris, 1998a). This hypothesis points to two requirements for long term changes in synaptic efficacy: (A) the local setting of tags at stimulated synapses and (B) the cell- or dendritic-domain wide availability of plasticity related proteins that can be captured by the tags and so enable mechanisms responsible for the stabilization of potentiation (Frey et al., 1988; Martin et al., 1997; Martin and Kosik, 2002; Scharf et al., 2002; Fonseca et al., 2004; Karpova et al., 2006; Reymann and Frey, 2007). The first part of this thesis replicates the results behind the heterosynaptic plasticity explained by the STC hypothesis. In addition to a mere replication, the experiments described in chapters 3 to 5 add a third synaptic pathway to the original setup design. This allows for a comparison of the potentiated pathways to a non-stimulated, control pathway that is fundamental for the assessment of long experiments.

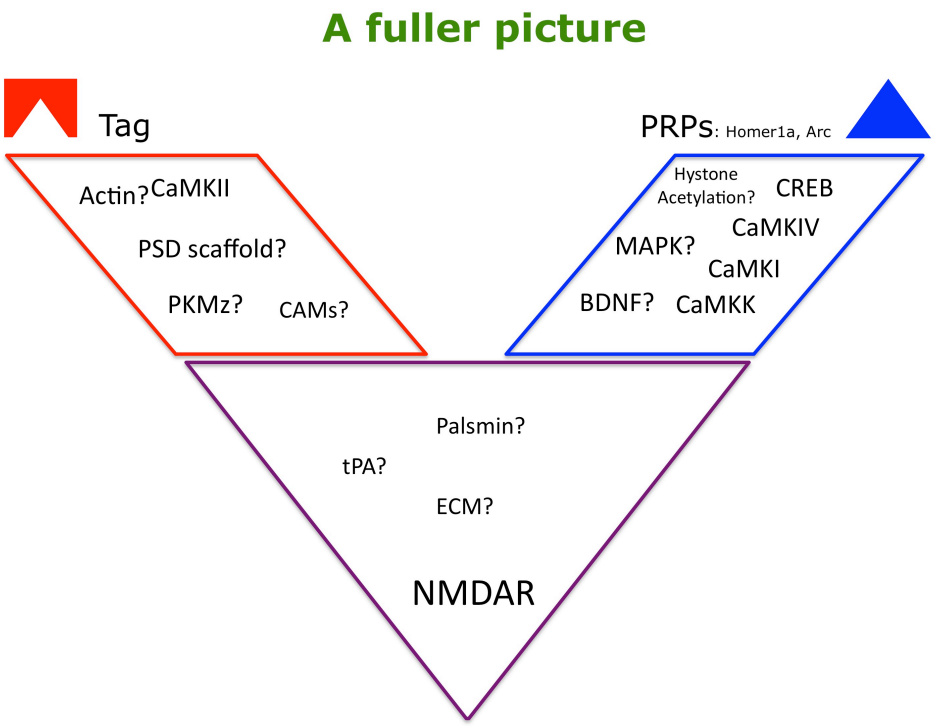
Amidst the potentially numerous molecular players and complex interactions involved in the processes involved in this form of heterosynaptic plasticity, some may be required for both tag setting and PRP availability (e.g. activation of N-methyl-D-aspartate receptors (O'Carroll and Morris, 2004)), whereas other molecules (or molecular states, such as phosphorylation) may only be necessary for tag setting, and yet others only for PRP synthesis and/or their availability (Figure 11.1). Part of this thesis involved the use of analytically appropriate stimulation protocols, explained in chapter 1, to identify components of the calmodulin kinase pathway involved in these two processes. I present evidence for a specific role in tag-setting of the CaMKII (Chapter 6) while another CaM Kinase, CaMKK (Chapter 7), has a specific role in the synthesis of PRPs. So, by working within the framework of the STC hypothesis, we have a clearer picture on what action certain kinases have in synaptic plasticity. The same techniques applied to the CaM Kinases here can be used, if the appropriate inhibitory drugs are available, on other molecules involved in

synaptic plasticity. Without defining the 'tag-block' and the 'PRP-block' experiments, as I have done in this thesis, other groups have used very similar protocols (i.e. dopamine (Sajikumar and Frey, 2004a) and NMDAR (O'Carroll et al., 2006). As more data is gathered on the roles of molecules in synaptic plasticity (see review in chapter 1), we have now enough information to propose, still within the STC framework, a new understanding of the processes involved in LTP.

Figure 11.1 The pyramid of molecular cascades.

The findings described in chapter 6 and 7 provide new information as to the role of two molecules (CaMKII and CaMKK) in the processes leading to the stabilization of synaptic change. CaMKII is a kinase whose activity is necessary for the setting of the tag. On the other hand, CaMKK is a kinase necessary for the successful synthesis and availability of PRPs. The theoretical framework provided by the STC hypothesis allowed for the development of of a conceptual diagram for the roles of particular molecules in LTP and LTD (Chapter 1).

Other molecules may have similar roles and these may be inferred from the literature available. Tests of 'tag-block' or 'PRP-block' should be carried out wherever the right drug is available in order to confirm these theoretical predictions.



11.4 The 'receptive' synapse model of synaptic plasticity

For more than ten years, experimental evidence has been interpreted within the framework of the STC hypothesis and what follows is an attempt to synthesize most of this information. I propose an updated view of the nature of the tag and its actions to enable the maintenance of synaptic change through the use of PRPs.

For too long, the problem of the identity of the tag has been approached as that of the identification of a single or a few molecules. A better knowledge of the complicated mesh of interactions at the synaptic level suggest an approach where the tag is understood as a necessary process with multiple components the failure of any of which would compromise the capture of PRPs and the maintenance of synaptic changes. At the same time, analytical and reductionist approaches add many molecules to the list of players in synaptic plasticity at the risk of leaving a complicated picture very hard to tackle by the other complementary tool in science (the synthesis of a theory). The updated STC model described here distinguishes between the mechanisms of expression of synaptic change and those responsible for the tag. Independently of the direction of synaptic change, I propose tagging to involve (i) an alteration of the spine architecture permissive and necessary for the remodelling of the PSD and (ii) the subsequent stabilization of the change in synaptic efficacy by the recruitment into the spine and the PSD of the stabilizing PRPs.

Some molecules required for the 'loosening' of the synapse are specific to the direction of the synaptic change (potentiation (CaMKII) or depression (Calcineurin)) and so are some of the PRPs required for the maintenance. The remodeling of the PSD-scaffolding and actin configuration allow these new products of protein synthesis to be added and maintain the structure supporting the expression of LTP and LTD (Lynch et al., 2007; Bramham, 2008; Cingolani and Goda, 2008). The end result is a change in the number of slots available for AMPAR insertion function (Lisman, 2003; Lisman and Raghavachari, 2006), and in the case of LTP, the perforation of the PSD (Geinisman et al., 1991) and the expansion or multiplication of the spine with both pre and postsynaptic modifications (Luscher et al., 2000).

What follows is an attempt to explain synaptic plasticity within the framework of this modified STC hypothesis. I propose a model in which the tag is understood as a required process of permissible synaptic change, without which novel protein synthesis is not allowed a role in the stabilization of that synaptic change (Figure 11.2). The molecular processes that account for the model are best depicted as snapshots at different times after LTP induction (Figures 11.3 to 11.6) and the descriptions follow the times attributed to those approximate time frames. The phenomenon of LTD and predictions of the model are described separately (section 11.4.6).

It is worth noting at the outset that the timing of the requirement for PRPs to stabilize the synaptic change is influenced by the experimental rate of test stimulation *in vitro* (Fonseca et al., 2006a). It is known that the rate at which new protein synthesis is required depends on the frequency of test stimulation (Fonseca et al., 2006a). When reading experimental results where LTP decays after 30 min or others when it decays after 2 hours, one has to take into account the frequency of test stimulation used by the experimenters. Slower frequencies (i.e. 0.006 Hz) of test stimulation delay the need for new PRPs while faster rates (i.e. 0.03 Hz) hasten the molecular turnover. Faster rates of stimulation can maintain kinases active and shorten the phases described for LTP therefore complicating the disentangling of the sequence of events involved. I have chosen some wide timeframes in order to fit literature researched under a variety of experimental conditions. The model is based on a stereotypical CA3 to CA1 connection and, for now, disregards differences between distal and proximal synapses (Andrasfalvy and Magee, 2004).

Figure 11.2 The 'receptive synapse' model of LTP.

The model is described in detail in the text. Briefly, the induction of LTP engages three parallel processes: (1) The expression of the synaptic potentiation, (2) the unlocking of the scaffolding holding together the PSD and the synapse, and (3) the synthesis of plasticity related proteins. Parallel to the initial expression mechanisms, the unlocking of the synapse into a 'receptive state' allows the gene products (both by local translation and somatic transcription) to reach their intended targets.

In the first 30 min after induction, the turnover of AMPAR has been shifted towards faster incorporation than removal by endocytosis (expression). Also, the scaffolding proteins holding PSD together have loosened allowing the expansion of the actin cytoskeleton (tagging).

Later, while the synapse is still in the receptive state (tagged), PRPs become available. They will:

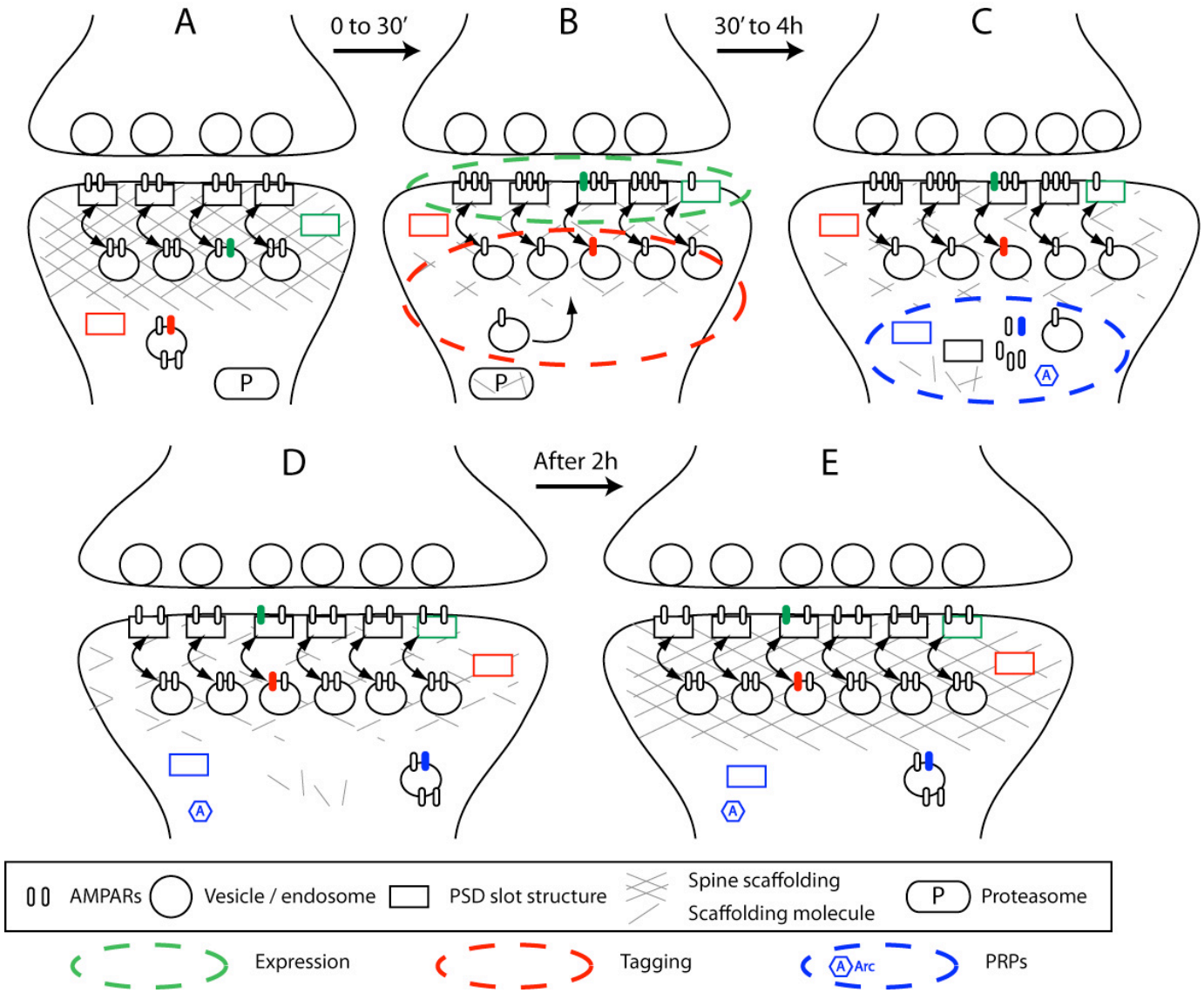
a) **Support the addition of new 'slots' for the incorporation of ionic receptors. Without the PRPs, the number of scaffolding molecules driven into the PSD in the early phases of LTP will decrease due to the standard molecular turnover of the synapse. Only with additional scaffolding molecules added to a reserve pool in the spine, the PSD can grow and sustain the synaptic change (see Fig. 11.7).**

b) **Prevent the return to prestimulation levels of AMPAR slots due to molecular turnover of AMPAR subunits. This is a consequence of point (a).**

c) **Build the presynaptic machinery responsible for the increase in vesicle release.**

d) **Further expand the spine through actin conformational changes.**

This allows for the expansion of the PSD, maybe of the spine, whith matching changes occurring presynaptically. Sometime after two hours, the synapse has reverted to a 'locked' state in which temporary changes in synaptic efficacy are allowed. However, unless the synapse is unlocked again, those changes (potentiation or depression) will not be stabilized.


A
0 to 30′
B
30′ to 4h
C
P
P
A
D
After 2h
E
A
A
AMPARs
Vesicle / endosome
PSD slot structure
Spine scaffolding
Scaffolding molecule
P
Proteasome
Expression
Tagging
A Arc
PRPs

11.4.1 Induction mechanisms (t = 0)

The arrival of action potentials at the presynaptic terminal depolarizes it, opening VGCC and allowing calcium in. This calcium increase is required to engage the SNARE protein complex and start vesicle fusion and the consequent release of glutamate onto the synaptic cleft (Rizo and Sudhof, 2002). During normal synaptic transmission glutamate binds to AMPARs and the consequent excitatory postsynaptic potential follows its course with no consequences for synaptic plasticity. However, if the postsynaptic membrane is depolarized at the time of glutamate release, the magnesium block onto the NMDAR is released and this receptor opens, allowing not only sodium ions but also calcium ions into the postsynaptic cell (Wigstrom and Gustafsson, 1986). Glutamate can also increase the concentration of cytosolic calcium via the activation of metabotropic glutamate receptors (class 1) whose action through phospholipase C (PLC) produces inositol 3-phosphate (I3P) that acts on its receptor (I3PR) in the endoplasmic reticulum (ER) releasing calcium from internal stores (Bashir et al., 1993). Ryanodine receptors also detect cytosolic calcium and respond by releasing ER calcium in a similar way to I3PRs.

Through multiple pathways, the increase in cytosolic calcium is sensed by calmodulin which, when bound to the cation, activates a series of processes and kinases including the calcium-calmodulin dependent kinase II (CaMKII) (Malenka et al., 1989). Ca-calmodulin releases the auto-inhibition of CaMKII and the autophosphorylated enzyme moves into the PSD (Shen and Meyer, 1999), out of reach of inhibitory phosphatases and closer to many of its targets (i.e. the NMDAR subunit NR2B) (Bayer et al., 2001; Bayer et al., 2006). The increase in cytosolic calcium also acts on calmodulin sensitive adenylyl cyclase (AC) and increases the levels of cAMP (Chetkovich and Sweatt, 1993), which are sensed by the cAMP-dependent protein kinase (PKA). Locally at the synapse, PKA can directly phosphorylate GluR1 (Esteban et al., 2003) and mediate the inhibition of phosphatases (PP1) that otherwise would block CaMKII (Makhinson et al., 1999).

PKA has a direct path into signalling to the soma and CREB phosphorylation (Bacskai et al., 1993; Impey et al., 1996; Nguyen and Woo, 2003) but it also has access to the protein synthesis machinery through its role in the activation of the

mitogen-activated protein kinase (MAPK), via the Ras-Raf pathway (Morozov et al., 2003). PKA is also responsible for the conversion of pro-neuropsin into neuropsin, which will have a role in the unlocking of the synapse at later time points (Matsumoto-Miyai et al., 2003). The pathway leading to the synthesis of PRPs requires the activation of PKA through receptors linked to adenyl cyclase (AC) (Sajikumar and Frey, 2004a). In addition to NMDAR dependent activation of adenyl cyclase, dopamine through D1/D5 receptors, noradrenalin through Beta-adrenoceptors (Thomas et al., 1996; Gelinas and Nguyen, 2005; Gelinas et al., 2008), and serotonin through the 5-HT receptor, all engage AC and activate PKA. We shall shortly discuss the importance of PKA activity in determining the direction of change in synaptic strength (Blitzer et al., 1995).

Outside the dendritic spine, the increase in cytosolic calcium is also sensed by calcium-calmodulin in the soma. Here, a depolarization capable of triggering action potentials will open voltage-gated calcium channels (VGCC). The somatic calcium increase activates CaM Kinase Kinase, which via CaM Kinase I and CaM Kinase IV (Bito et al., 1996), engages the upregulation of PRPs later necessary for the maintenance of LTP (Raymond, 2008).

At this initial time point, other events are taking place parallel to those triggered by the increase in intracellular calcium. mGluRs engage phospholipase A2 and this allows the release of arachidonic acid (AA) from the postsynaptic membrane to the synaptic cleft. AA will act as a retrograde messenger later in this model (Williams et al., 1989). A similar role as retrograde messenger can be attributed to nitric oxide (Christopherson et al., 1999; Taqatqeh et al., 2009). Also, the activation of phospholipase C (PLC) by mGluRs and the muscarinic acetylcholine receptor (mAChR) leads to the activation of PKC through the increase in diacylglycerol (DAG). PKC, as PKA, converges onto the MAPK pathway via its actions on Raf (Roberson et al., 1999).

At this stage, right after synaptic stimulation, there is an intact extracellular matrix (ECM) and cell adhesion molecules (CAM) holding the distances between pre and postsynaptic membranes as well as between them and glial cells. Intracellularly, molecules in the PSD are locked together by scaffolding molecules (PSD95, GKAP,

Homer) and the PSD holds the shape of the synaptic spine via its link to the actin cytoskeleton (SPAR, Shank-cortactin).

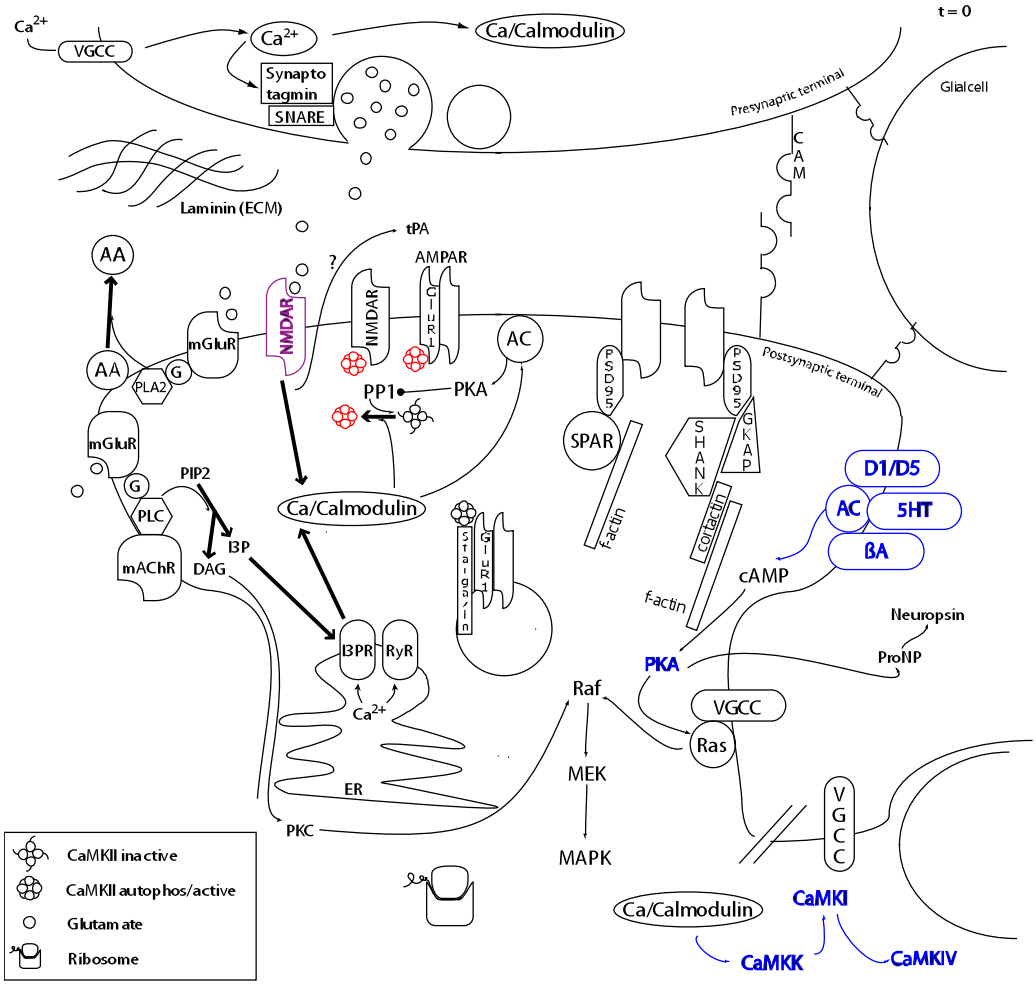
Figure 11.3 Induction of LTP.

Induction of LTP: Pre and postsynaptic events taking place right after the induction of LTP by glutamate release in a typical Schaffer collateral – CA1 pyramidal cell synapse. The steps illustrated in this figure are described in detail in the text. The original state of the synapse at rest is represented by a compact extracellular matrix (ECM) with cell adhesion molecules linking pre and postsynaptic membranes, as well as an organized and lined up actin cytoskeleton and PSD scaffold.

The release of neurotransmitters, through ionic or metabotropic receptors, triggers the activation of kinases and the release of retrograde messengers. The effect of these activations is described in the next stage of the model.

In colour, three examples of the three possible roles in the processes behind the STC hypothesis. In purple, the NMDAR, without the opening of which, there is no tag setting nor synthesis of PRPs (colours match those of scheme in Figure 11.1). In red, autophosphorylated CaMKII, necessary for the setting of the tag but not for the synthesis of PRPs, as elucidated in the main work of this thesis. In blue, D1/D5 receptor activation, shown to be necessary to make PRPs available but not to set the synaptic tag (Sajikumar and Frey, 2004a). The results presented in chapter 7 of this thesis support a necessary role in the synthesis of PRPs for the CaMKK pathway (also in blue).

Abreviations: VGCC: voltage gated calcium channels; AA: Arachidonic Acid; PLA: Phospholipase A; PLC: Phospholipase C; mGluR: metabotropic glutamate receptor; tPA: tissue plasminogen activator; AC: Adenylyl cyclase; DAG: diacyl glycerol; I3P: Inositol-3-phospate; RyR: Ryanodine Receptor; PKA: Protein Kinase A; βA: Beta-adrenergic receptor; ProNP: Proneuropsin; CAM: Cell adhesion molecule, PP1: Protein phosphatase 1; ECM: Extracellular Matrix; ER: endoplastic reticulum; 5HT: Serotonin (5-hydrosytryptamine) Receptor; MAPK: Mitogen-activated protein kinase; PIP2: Phosphatidylinositol 4,5-biphosphate; PKC: Protein Kinase C; mAChR: metabotropic AcetylCholinergic Receptor.


t = 0
Ca2+
VGCC
Ca2+
Ca/Calmodulin
Synapto tagmin
SNARE
Presynaptic terminal
Glialcell
CAM
Laminin (ECM)
tPA
AA
?
AMPAR
NMDAR
NMDAR
GluR1
mGluR
AC
AA
PLA2
G
PP1
PKA
PSD95
PSD95
Postsynaptic terminal
mGluR
SPAR
SHANK
GKAP
G
PIP2
D1/D5
PLC
Ca/Calmodulin
AC
5HT
Stargazin
GluR1
ßA
mAChR
DAG
IP3
f-actin
cortactin
cAMP
f-actin
Neuropsin
IP3R
RyR
PKA
ProNP
Raf
Ca2+
VGCC
Ras
ER
MEK
PKC
MAPK
VGCC
CaMKI
Ca/Calmodulin
CaMKK
CaMKIV
CaMKII inactive
CaMKII autophos/active
Glutamate
Ribosome

11.4.2 Expression and tagging (t = 0 to 30').

Minutes after the synaptic input capable of raising intracellular levels of calcium, an autophosphorylated form of CaMKII remains active in the PSD even after the calcium levels, detected by calcium calmodulin, revert to baseline levels (Yamauchi and Fujisawa, 1985; Yoshimura and Yamauchi, 1997). In this state, CaMKII binding to the NR2B subunit of the NMDAR becomes persistent (Bayer et al., 2006) and this keeps CaMKII in the postsynaptic density, decreasing the inhibitory effect of some phosphatases on CaMKII (Strack et al., 1997a; Yoshimura et al., 1999; Fox et al., 2006). CaMKII bound in the PSD can still be inactivated and released from the PSD by dephosphorylation by phosphatases (i.e. PP1) (Strack et al., 1997b; Yoshimura et al., 1999). PKA activity may prevent or reduce this inhibitory effect (Strack et al., 1997a). Once in the PSD, CaMKII phosphorylates more than 28 substrates (PSD-95, tubulin, neurofilaments, glutamate receptor subunits, among others) (Yoshimura et al., 2000). One of these substrates, the PSD transmembrane protein densin-180, binds with enhanced affinity to the autophosphorylated form of CaMKII, also helping the kinase to localize to the PSD (Walikonis et al., 2001).

As mentioned before, CaMKII enhances Ca^{2+} influx through the NMDAR (Kitamura et al., 1993). CaMKII also increases the AMPAR GluR1 subunit's conductance (McGlade-McCulloh et al., 1993; Derkach et al., 1999) and phosphorylates the AMPAR associated protein stargazin, which promotes the incorporation of AMPAR into the PSD (Hayashi et al., 2000; Tomita et al., 2005). Indeed, the levels of AMPARs seem to increase for at least the first 20 min after glutamate uncaging (Bagal et al., 2005). CaMKII also acts on the endocytic protein dynamin (Yoshimura et al., 2000). Dynamin has a fundamental role in the pinching off of the endocytic vesicle from the membrane (Urrutia et al., 1997). Although the effect of CaMKII on dynamin is unknown, its phosphorylation by another kinase, ERK, inhibits the dynamin-microtubule interaction necessary for vesicle endocytosis (Earnest et al., 1996). Maybe, CaMKII phosphorylation of dynamin also inhibits its endocytic function. The combined effect of CaMKII on stargazin and dynamin would disturb the balance of receptor turnover towards faster incorporation and slower removal. Overall, the expression of LTP seems to be primarily postsynaptic at

this early stage (Bayazitov et al., 2007) and can be explained by an increase in the number of AMPARs and an enhancement of their function (Poncer, 2003).

Concomitant to the changes that directly affect synaptic strength, CaMKII activity contributes to the expansion of the actin cytoskeleton. In fact, CaMKII activity may not be necessary for the expression of LTP as KN-93 and AIP experiments still allow for the incorporation of AMPARs and E-LTP (chapter 6 and others (Sajikumar et al., 2007)). However, CaMKII seems to be necessary for the induction and maintenance of the receptive state for the incorporation of PRPs (tagging). How does CaMKII loosen the PSD scaffolding and expand the cytoskeleton?

CaMKII may act by activating the Ras-GTPase SynGAP, which inactivates Ras which, through multiple steps of kinase activations (Rac, PAK, LIMK) and will now fail to phosphorylate cofilin (Carlisle and Kennedy, 2005). The resulting unphosphorilated and active cofilin can now severe F-acting (Carlisle et al., 2008b). This disruption of the actin cytoskeleton downstream of CaMKII contrasts with the stabilizing effect of CaMKII on the polymerization of F-actin reported in live imaging of actin dynamics within single spines (Matsuzaki et al., 2004; Honkura et al., 2008). Maybe the role of CaMKII is different depending on which pool of actin it is exerting its funcion (Cingolani and Goda, 2008). There may be a dynamic pool of actin very close to the PSD that needs to be loosened to allow for the expression of LTP while another 'enlargement' pool is responsible for the expansion of the spine and requires CaMII-mediated stabilization (Bramham, 2008; Honkura et al., 2008).

Besides the role of CaMKII, focus of this thesis but not this model, there are other ways in which synaptic stimulation can alter the actin conformation that holds the PSD and the dendritic spine together. Following glutamate stimulation, the scaffolding molecule cortactin is moved out of the spine into the dendritic shaft (Hering and Sheng, 2003), allowing for actin reconfiguration (Halpain et al., 1998). On the other hand, some proteins move closer to the PSD after NMDAR activation (Sharma et al., 2006). Among them, Homer1a protein is shipped into the spine (Okada et al., 2009) on its way towards the PSD. Homer1a will have an important role in the stabilization of a new, expanded PSD and spine later on.

Another player in the unravelling of the scaffolding of the spine and the PSD is the proteasome and the protein degradation without which, the maintenance of LTP is

not possible (Lopez-Salon et al., 2001; Hegde, 2004; Fonseca et al., 2006b; Dong et al., 2008). In Dong et al. (2008), the inhibition of the proteasome enhances the expression of E-LTP while impairing the maintenance of L-LTP. This is another example of the dissociation between the expression of LTP and the tagging of the synapse or the synthesis of PRPs. The enhancement of the initial expression of LTP during proteasome inhibition is due to the reduced degradation of new proteins translated locally and this is independent of whether tags are set or PRPs are transcribed (Dong et al., 2008). The degradation of scaffolding proteins, the initial disentanglement of the PSD and expansion of the actin network around it, are necessary steps for the later stabilization of the new synaptic configuration. The actions of the proteasome are depicted in figures 11.2, 7, 8 & 9 (model figures) but are not included in figures 11.3 to 6.

PKA plays a necessary role in the maintenance of the NMDAR-dependent enlargement of the spine, probably by inhibiting phosphatases (Yang et al., 2008). Interestingly, in the same way that LTP can be expressed without spine expansion (i.e. without tag formation), the inhibition of exocytosis or PKA activity can block the expression while allowing for a temporary expansion of the spine (i.e. tag without LTP) (Yang et al., 2008). We shall discuss the relevance of this 'silent tagging' when explaining the slow-onset plasticity after chemical LTP and replay of place cell activity (Isaac et al., 2009).

Another event taking place at this time is the temporary break down of the physical attachment between pre- and postsynaptic membranes, as well as that of glial cells. Many of the following steps may be necessary for the setting of the tag but not for the expression of LTP. The release of proneuropsin triggered by PKA allows for enough neuropsin to cut loose the extracellular matrix (ECM) protein laminin as well as the cell-adhesion molecule (CAM) L1 (Komai et al., 2000; Matsumoto-Miyai et al., 2003). Also acting on the same ECM, plasminogen is converted into active plasmin by tPA and this plasmin breaks up laminin filaments (Mizutani et al., 1996; Nakagami et al., 2000). Cell adhesion molecules are internalized and their intercellular contacts broken, as seen after the serotonergic induction of facilitation in in *Aplysia* (Bailey et al., 1992; Mayford et al., 1992). All this allows for the presynaptic and the postsynaptic membrane, as well as glial cells

around the synaptic cleft, to break their close contact and unlock the synapse for further changes.

Presynaptically, there are a series of events, dependent on postsynaptic calcium entry that will contribute to the expression of synaptic changes. Independently of what retrograde messenger is involved in the communication between postsynaptic spine and presynaptic axonal bouton, presynaptic changes are critical to the expression of synaptic plasticity (Enoki et al., 2009) and act together with postsynaptic modifications (Lisman and Harris, 1993). Presynaptic changes are dependent on the activation of VGCC and the retrograde signalling downstream of postsynaptic PKA, probably via arachidonic acid (Bayazitov et al., 2007). For every GluR1 subunit added postsynaptically, the vesicle-associated protein synaptophysin is added on the opposite side of the synaptic cleft, probably pairing AMPAR clusters with vesicle release sites (Antonova et al., 2001). Synaptophysin and other scaffolding and adhesion molecules may be responsible for the anatomical pairing of the presynaptic active zone with the postsynaptic PSD (Lisman and Harris, 1993). Presynaptically we find changes in the properties of ionic channels, in the composition of the molecular scaffold that supports them, and in the phosphorylated state of kinases and other effector proteins. The end result is an increase in the quantal content of every action potential (Bolshakov et al., 1997).

Simultaneous to the changes required for the immediate expression of LTP and the unlocking of the synapse, there is a third series of events, these leading to the synthesis of PRPs, both through local translation and nuclear transcription. In the first minutes after induction mRNAs already present in the dendrites are translated. The PRPs generated by local translation, however important for the maintenance of LTP, as they are not speculated to move far along the denrtitic tree, cannot account for the heterosynaptic plasticity described by the STC hypothesis. Still, during local translation ribosomes that rest in the dendritic shaft are moved into stimulated spines (Ostroff et al., 2002). In the dendritic spine, a series of kinases phosphorylate the cytoplasmic polyadenylation element (CPE) binding protein (CPEB), which activates CPE and this, in turn, adds poli-adenosine tails to mRNAs already present at the spine. This poly-A tail marks mRNA for translation. The MAPK pathway is necessary to enable local translation (Kelleher et al., 2004a; Gong and Tang, 2006)

while other kinases, (i.e. CaMKII and Aurora) can also influence CPEB phosphorylation (Huang et al., 2002; Atkins et al., 2005). The participation of so many kinases opens the possibility for some redundancy. As reported in this thesis (Chapter 6), the lack of a necessary role for CaMKII in the synthesis of PRPs concerns PRPs shipped around the dendritic trees and those experiments cannot attribute any role to CaMKII in local, synapse-specific translation. As mentioned previously, SNK mRNA is translated at this time, together with tissue plasminogen activator (tPA), CaMKII (Scheetz et al., 2000), actin and Arc, among others (Steward and Schuman, 2001, 2003). The roles of these diverse products of translation will be discussed as their roles become relevant to the storyline of synaptic change. These local PRPs are nevertheless not sufficient to maintain the late phase of LTP and do not provide an answer to the problem of the targeting of the new PRPs or mRNAs synthesized in the soma, necessary for the STC heterosynaptic plasticity.

Parallel to local translation of mRNAs, the signal for somatic transcription may reach the nucleus after calcium entry via the recruitment of MAPK via Rap1/Ras GTPase pathway (Morozov et al., 2003). This triggers the acetylation of histone 3 which in turn enables initiation factors to access promoter regions (CRE and SRE (Davis et al., 2000)) of plasticity related genes. A possibly faster kinase pathway dependent on the entry of calcium, that of CaMKK and CaMKIV (chapter 7), reaches similar targets as MAPK (CREB serine 133) and will also contribute to the transcription of new mRNAs (Hardingham et al., 1998; Hardingham et al., 1999; Wu et al., 2001; Deisseroth et al., 2003).

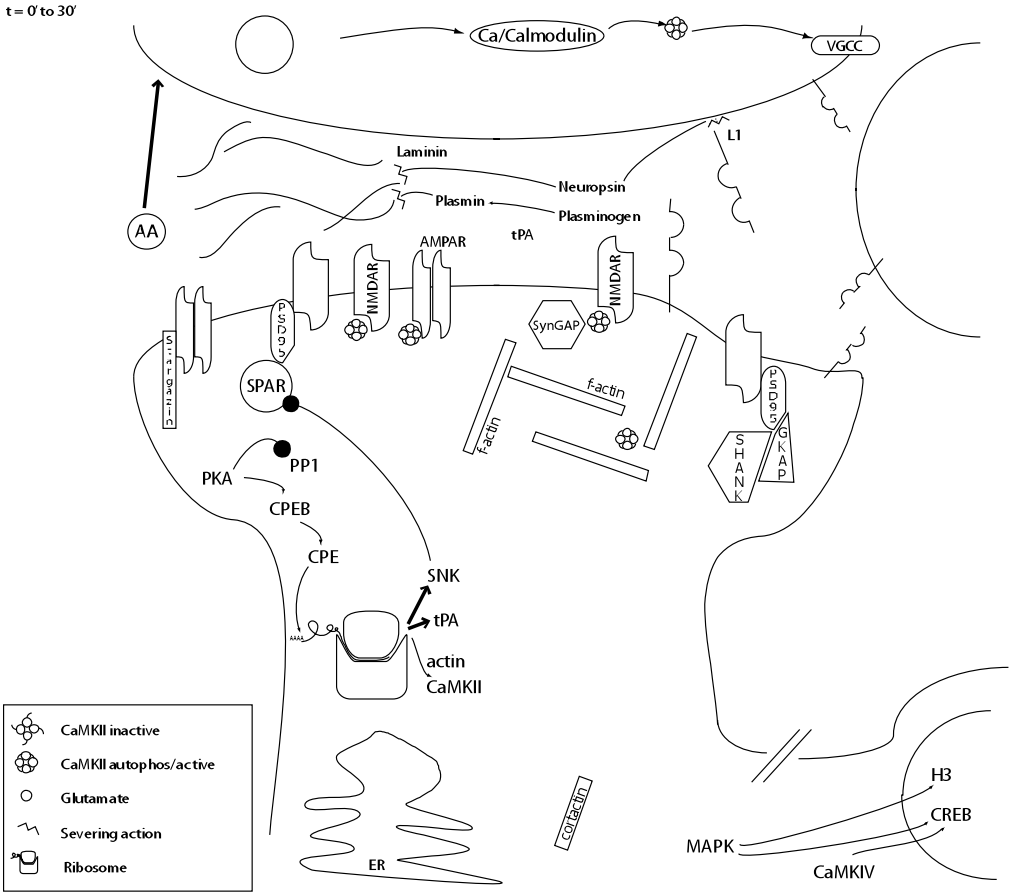
Figure 11.4 Three parallel processes in the early stages of LTP.

1. **Expression of LTP: AMPAR conductances are enhanced by CaMKII phosphorylation. At this stage, the effect of CaMKII on stargazin has shifted the balance of AMPAR receptor turnover towards greater AMPAR insertion in the PSD. MAPK changes the properties of potassium channels reducing the decay constant of EPSC propagation through the dendrite. Presynaptically, CaMKII also changes the properties of VGCC enhancing neurotransmitter release.**

2. **Unlocking of the synapse (tagging): CaMKII contributes to the expansion of the actin cytoskeleton. The scaffold molecule cortactin is moved out of the PSD while the proteasome degrades other scaffolding molecules. In the synaptic cleft, the actions of neuropsin and plasmin on cell adhesion molecules and the extracellular matrix loosen the connection between pre and postsynaptic membranes.**

3. **Signal for PRP synthesis: In the soma, CaMKIV and MAPK engage de novo protein synthesis. At the dendrites, ribosomes are starting translation.**

Abreviations: CPE: cytoplasmic polyadenylation element; CPEB: cytoplasmic polyadenylation element binding protein; VGCC: voltage gated calcium channels; ER: endoplasmic reticulum, AA: Arachidonic Acid; PLA: Phospholipase A; PLC: Phospholipase C; tPA: tissue plasminogen activator; AC: Adenylyl cyclase; DAG: diacyl glycerol; I3P: Inositol-3-phospate; RyR: Ryanodine Receptor; PKA: Protein Kinase A; βA: Beta-adrenergic receptor; PP1: Protein phosphatase 1; MAPK: Mitogen-activated protein kinase.


t = 0' to 30'
Ca/Calmodulin
VGCC
L1
Laminin
Neuropsin
Plasmin
Plasminogen
tPA
AA
AMPAR
NMDAR
NMDAR
PSD95
Stargazin
SynGAP
SPAR
f-actin
f-actin
PSD95
SHANK
GKAP
PKA
PP1
CPEB
CPE
SNK
tPA
actin
CaMKII
CaMKII inactive
CaMKII autophos/active
Glutamate
Severing action
Ribosome
ER
cofactin
H3
CREB
MAPK
CaMKIV

11.4.3 Expansion (t = 30' to 4 h)

Two hours after stimulation, the synapse is in a stage at which expression of LTP has been maintained due to the insertion or removal of glutamate receptors, as well as changes in their properties. During this period, the postsynaptic mechanisms of expression are starting to be replaced by presynaptic changes (Bayazitov et al., 2007). Independently of the location of the expression of synaptic changes, a series of events may have prepared the dendritic spine for the receiving of gene products, without which the changes in expression will revert back to pre-stimulation levels. In the 'receptive synapse' model of STC, there are multiple non-exclusive ways in which a synapse 'remembers' its pre-potentiation strength. (i) The mechanisms of expression have changed the balance in the dynamic turnover of receptors and scaffolding molecules in the PSD towards a state where most components of the machinery responsible for the expression of LTP have been incorporated into the PSD. This lowers the molecules in the reserve pool present at the spine and unless this pool is reinforced with new PRPs, the balance will be restored as soon as the kinases responsible for the expression of the potentiation cease to act; or (ii) the new molecules responsible for the enhancement in synaptic efficacy may require PRPs to cement their position in the PSD and reach a state that only those molecules involved in basal synaptic transmission enjoyed. Without the consolidation of the new 'hyperslots' (Lisman and Raghavachari, 2006), the synapse falls back to a configuration that was already stable before the induction of LTP.

The necessary PRPs may not be available to the potentiated synapse because they were never synthesized, in which case the receptive (tagged) synapse has nothing to capture (Fig. 11.7 upper panel). Or the PRPs may be available and distributed along the dendrites but the synapse is found locked (untagged) and consequently, the PRPs cannot reach their site of action (Fig. 11.8). To keep the synapse tagged (unlocked), the switch properties of certain kinases (CaMKII) keep the PSD scaffolding open and the actin cytoskeleton expanded allowing the incorporation of new molecules and gene products (PRPs). One of these PRPs is the autonomously active kinase PKMζ, which will critically contribute to the incorporation of AMPAR into the PSD (Ling et

al., 2006). Due to its mechanism of action, PKMζ is necessary for LTP maintenance but not for LTD maintenance. This is the first LTP specific PRP identified (Sajikumar et al., 2005c).

There are other PRPs with a role in expanding the PSD and maybe the spine. New actin filaments grow the dendritic spine and are stabilized by profilin (Ackermann and Matus, 2003). Homer1a and other scaffolding molecules have room to start linking the new machinery with support structures like the ER (Ehlers, 2002). Briefly, Homer1a binds to mGluRs (Brakeman et al., 1997) and to PSD-95 complexes through Shank proteins (Tu et al., 1999). Homer1a also links this PSD machinery to rynaodine and I3PRs in the ER (Xiao et al., 2000) and Shank recruits Homer1a into the PSD (Sala et al., 2001). At about the same time, SPAR (Rap-GTPase-activating protein (RapGAP)) reorganizes the actin cytoskeleton linking F-actin to NMDARs (Pak et al., 2001). An active Ras pathway will also inhibit cofilin and this will stop the severing of actin (Carlisle et al., 2008b). Finnally, Kopec et al. (2007) show that the incorporation of the GluR1 subunit of the AMPAR into the PSD supports the new expanded structure via its C-tail (Kopec et al., 2007). However, they show that GluR1 incorporation is not enough to expand the spine as it needs NMDAR activation and calcium influx. Kopec et al., 2007 conclude that "LTP initiates two parallel pathways, one leading to an increase in synaptic strength through the exocytosis and synaptic insertion of GluR1 and the other to an increase in spine size through reorganization of the actin cytoskeleton. Each pathway may be initiated independently but become interdependent for long-term stabilization." Their study provides a fundamental piece to the puzzle of LTP by firstly, distinguishing between the expression of LTP and the structural changes responsible for its maintenance and secondly, linking the two processes through the properties of the GluR1 subunit of the AMPAR. The 'receptive synapse' model also dissociates the expression of synaptic changes from the structural malleability necessary to explain the heterosynaptic plasticity behind the STC hypothesis. Some of the scaffolding molecules are newly synthesized PRPs obtained through local translational processes. Later, somatic gene products like Homer1a will arrive to the dendrites and replenish the pool of proteins used both for the expression and the stailization of the synapses (Okada et al., 2009). Without a receptive and expanded synapse (tagged),

these scaffolding molecules have nothing to stabilize or lock. In this situation, even if LTP is expressed due to an increase in AMPARs in the PSD and a change in their conductance properties, the lack of a support structure, both presynaptically and postsynaptically, will eventually lead to a reversion towards the synaptic configuration present before LTP induction (Fig. 11.8). In other words, the new translation and transcription products are necessary for the conformational changes in the spine as well as the expression of the change in synaptic efficacy (Yang et al., 2008). However, the PRPs will not find room or access to a closed up, static, locked PSD (i.e. untagged), while they will be used and captured by open, expanded, unlocked PSDs (i.e. tagged).

If the PRPs are allowed to act by a receptive synapse, PSDs can be seen dividing (perforated) forty minutes after stimulation specifically in the synapses that received the stimulation (Buchs and Muller, 1996). Even new spines can emerge 60 min after LTP induction (Engert and Bonhoeffer, 1999). The loosening of the extracellular matrix and cell adhesion molecules may also enable the increased glial coverage seen after synaptic potentiation (Lushnikova et al., 2009). The 'receptive synapse' model predicts that the structural changes that unlock the PSD take place independently of the direction of change in synaptic efficacy that a particular synapse is experiencing. This could account for the cross-tagging properties of the STC hypothesis and is discussed below (Fig. 11.7).

As the synapses grow, the ER provides a source of membrane phospholipids. This postsynaptic exocystosis is required for the expansion of the dendritic spine and for the expression of LTP (Yang et al., 2008). Also, newly transcribed mRNAs are shipped through the microtubule network (Kohrmann et al., 1999) into the ER (Wickham et al., 1999), which will then translate, process and transport proteins deep into the dendrites (Gardiol et al., 1999; Maas et al., 2009). There is still no clear answer as to why some gene products are always present in the dendrites as mRNAs while others are only transported there after the induction of synaptic plasticity (Martin et al., 2000a). There seems to be, nevertheless, no targeting of these products into particular synapses. The 'receptive synapse'model attempts to solve the targeting puzzle by dismissing its need altogether. The kinases responsible for the expression of either change in synaptic strength will maintain a receptive state at the

synapse and consequently recruit the necessary gene products as long as the synapse is unlocked, tagged for change.

Presynaptically, 20 min after LTP induction there are more vesicles attached to the active zones while the overall vesicle density decreases due to the expansion of the spine area (Applegate et al., 1987; Meshul and Hopkins, 1990). In the dentate gyrus, presynaptic proteins syntagmin, synaptophysin and synaptotagmin are more concentrated after 3 hours but not after 45 min (Lynch et al., 1994). Another type of presynaptic change is expressed as changes in the properties of VGCC after their modulation by CaMKII. Vesicle release relies on the interaction between syntaxin and SNAP-25 with the VGCCs. The synprint peptide binds to syntaxin preventing this interaction unless active CaMKII phosphorylates synprint thereby blocking synprint's inhibition of the release machinery (Yokoyama et al., 2005). To summarize, presynaptically we find active zones, scaffolding molecules regulating channel numbers and associated kinases like CaMKII all acting in a very similar way as those molecular players in control of synaptic plasticity postsynaptically (Catterall and Few, 2008).

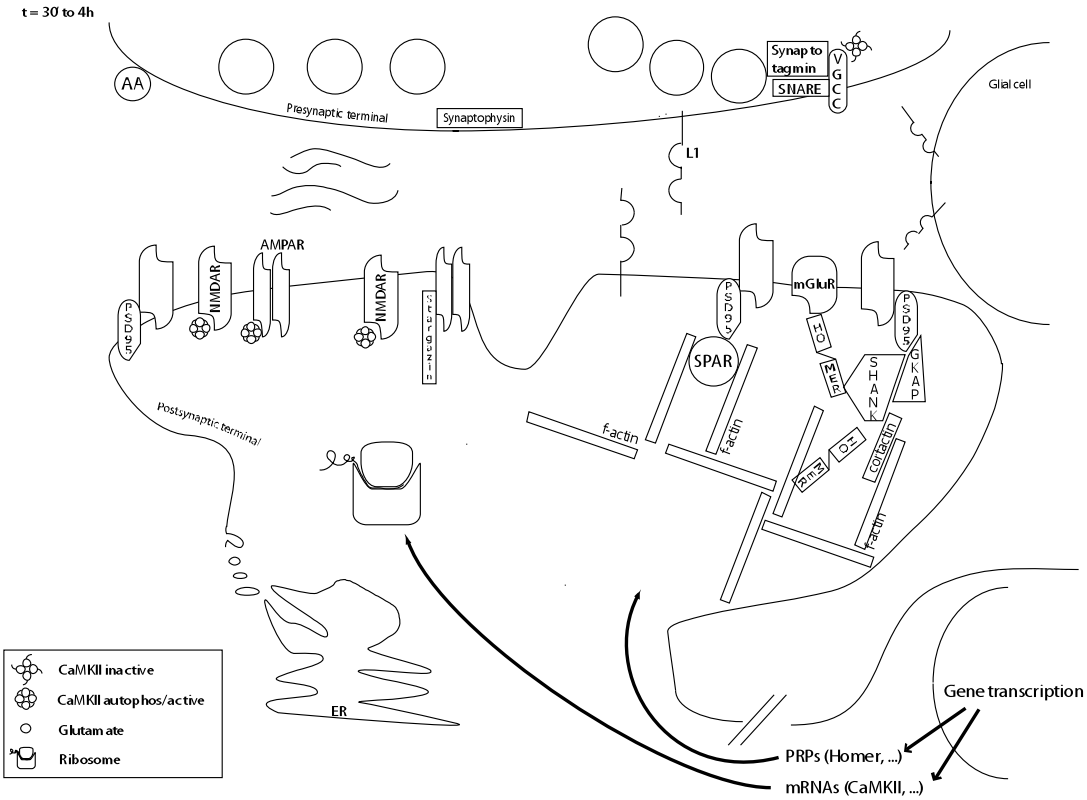
At some point towards the end of this stage, CaMKII may finally succumb to the effect of phosphatases (PP1) (Yoshimura and Yamauchi, 1997; Yoshimura et al., 1999). The fading activity of particular kinases may account for the limited lifespan of synaptic tags (Frey and Morris, 1998b). There is the possibility that some CaMKII remains active deep in the PSD (Sanhueza et al., 2007). However, most other inhibitors of CaMKII support the view that this kinase is not necessary for the maintenance of LTP (Malinow et al., 1989; Otmakhov et al., 1997; Bortolotto and Collingridge, 1998; Chen et al., 2001). Additionally, the inhibitory actions of PP1 on CaMKII may be enhanced at this later stage by a decreased activity of PKA on inhibitor 1 (Strack et al., 1997a). After the decay in CaMKII activity, a synapse that has received PRPs will have stabilized it new structural conformation and maintain its change in synaptic efficacy.

Figure 11.5 Stabilization of synaptic changes.

The new PRPs find an unlocked synapse and are capable of performing their function. Some of them are scaffold proteins that will add new 'slots' to the PSD and maybe expand the spine. There will be more Homer1a, F-actin, cortactin, and other scaffold molecules finding their way to the PSD.

Some other molecules will need replenishing in order to sustain the increased demand of an enlarged synaptic connection (CaMKII, AMPAR subunits, …).

Postsynaptic changes are matched presynaptically through the pairing of synaptophysin to the new postsynaptic AMPARs 'slots'. In this way, new vesicles are moved closer to the membrane. Additionally, more CaMKII increases vesicle release probabllility through synprint inhibition. With the help of the endoplasmic reticulum and a loose extracellular matrix, the two sides of the synapse grow.



11.4.4 Locking up and stabilization of synaptic plasticity (t > 4 h)

This is the final picture after local synaptic processes and cell-wide protein synthesis have been allowed to interact. The synapse, after the incorporation of gene products, has now expanded, maybe adding an additional PSD or splitting up the dendritic spine into two. The extra Homer1a protein links a variety of components of the PSD and SPAR, Shank and cortactin act as scaffold molecules stabilizing the actin cytoskeleton. New receptors have been added to the PSD and, even under turnover, the same number of 'hyperslots' (Lisman and Raghavachari, 2006) are kept occupied thanks to the new configuration of scaffolding molecules and the additional proteins synthesized *de novo*. One of these newly added proteins, the GluR1 subunit of the AMPAR, not only contributes to the expression of LTP bu also stabilizes the enlarged PSD (Kopec et al., 2007). The ER expands to keep acting as a source of intracellular calcium release while ribosomes and translation return to a pre-tetanization state.

Most importantly, the scaffolding returns to a locked state in which it allows temporary changes in synaptic efficacy but will remember this new configuration and revert to it unless a new process of untangling is accompanied by new PRPs (i.e. new strong stimulation unlocks the synapse). Extracellularly, new CAMs have linked the expanded synapse and the extracellular matrix also reverts to its compact state. Connecting pre and postsynaptic changes, adhesion molecules are produced and recruited to synaptic sites. For example, N-cadherin is synthesized after PKA activation and is required for the maintenance of LTP but not for the expression of its earlier phases (Bozdagi et al., 2000). N-cadherin could act as the cement connecting the two sides of new active zones. This would stabilize the novel interactions between synaptophysin and GluR1 (Antonova et al., 2001). The late presynaptic changes seem capable of taking over the maintenance of LTP from the postsynaptic changes (Bayazitov et al., 2007). Although at quite fast test stimulation rates, Bayazitov et al., 2007 detect an early postsynaptic component in the expression of LTP that within 2 hours has switched completely to a presynaptic increase in vesicle release.

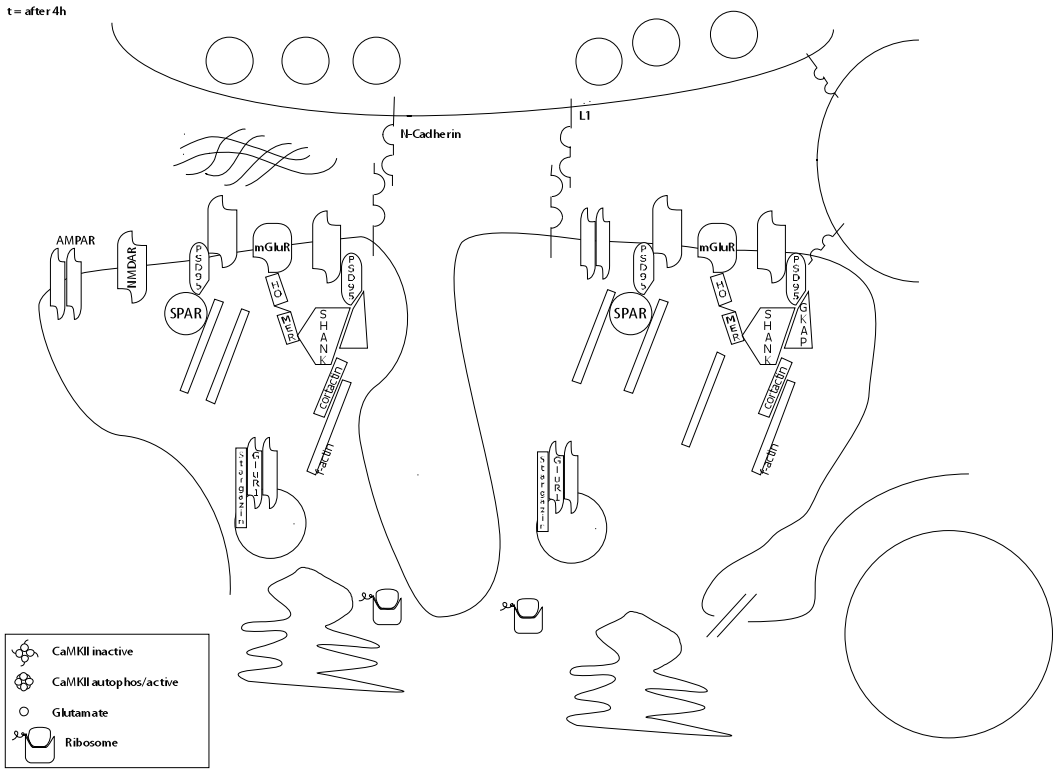
This new locked state may still require some constant activity of kinases (CaMKII and PKMζ) for some local events (receptor phosphorylation) but overall, the locked synapse could be stable with low energy expenditure.

Figure 11.6 Final stage in the consolidation of synaptic changes.

The synapse returns to a locked state where the scaffold sustaining the PSD is all in place, the actin cytoskeleton is in a dynamic but stable state, and the synaptic cleft has a compact extracellular matrix and enough of cell adhesion molecules to cement the new conformation.

Sustaining the expression of LTP there are new 'slots' for AMPAR with presynaptic matching vesicle release sites. The PRPs not only add to the spine but also replenish the pool of molecules in constant turnover. This keeps a constant synaptic strength even in a dynamic state.

New changes in synaptic efficacy will be expressed by the addition or the removal of AMPAR but the change will only be maintained if the synapse is unlocked and new PRPs are produced in order to stabilize those changes.



11.4.5 STC explained by the receptive synapse hypothesis

The sequence of events described above is based on a wide range of literature on LTP but crucially, on clues discovered by the pairing of heterosynaptic stimulation of different strengths. In 1997, Frey and Morris described a property of LTP revealed by the combination of strong (L-LTP inducing) and weak (E-LTP inducing) heterosynaptic stimulation (Frey and Morris, 1997). The surprising result, that E-LTP is rescued into L-LTP through the products of protein synthesis made available by the strong heterosynaptic stimulation, is best explained by the synaptic tagging and capture hypothesis (Frey and Morris, 1998a). The identity and properties of the tag have been studied since in an attempt to better understand the molecular basis of learning and memory (Martin and Kosik, 2002; Fonseca et al., 2004; Navakkode et al., 2004; Sajikumar and Frey, 2004a, b; Zhong and Zucker, 2004; Navakkode et al., 2005; Sajikumar et al., 2005c; Young and Nguyen, 2005; Alarcon et al., 2006; Young et al., 2006; Lopez-Rojas et al., 2007; Reymann and Frey, 2007; Sajikumar et al., 2007; Viosca et al., 2007; Okada et al., 2009). In this brief review, I propose an updated understanding of the tag as a process and not a molecule or a set of molecules. This process consists of the unlocking of the molecular scaffolding holding the PSD and the synaptic spine. The quest for the identity of the tag is also re-formulated as a quest to find molecules with a necessary role for the unlocking of the PSD (chapter 1 section 1.4.4). The PRPs and their capture, as expected, are necessary for the stabilization of the changed PSD but can only act on unlocked, receptive spines. The details of the expression, unlocking and locking of LTP have been described in previous sections. In this section, I attempt to explain the experimental observations that lead to the formulation of the STC hypothesis under a new 'receptive synapse' version of the model.

E-LTP being expressed at one set of synapses can be rescued into L-LTP by the PRPs made available by heterosynaptic induction of LTP (Frey and Morris, 1997), by increased levels of cAMP (Frey et al., 1993; Navakkode et al., 2004), by the expression of constitutively active CREB (Barco et al., 2002) and basically, by a battery of ways that engage the synthesis of PRPs. Following the explanation of LTP by the 'receptive synapse' model described above, an unlocked synapse (tagged) will

only be capable of maintain new slots for the inclusion of AMPARs, and maybe open new puncta in the PSD, if additional PRPs reach the synapse in this receptive state. Without the provision of PRPs, the scaffold will lock again as the activity of kinases responsible for tagging fades (CaMKII in LTP, maybe Calcineurin in LTD). PRPs will have no possibility of being added to the PSD if they arrive after the locking of the spine. Some early experiments attempted to measure the lifetime of the tag, that is, the time window around the induction of LTP in which PRPs can be captured and LTP stabilized. Indeed, it looks as if synapses experiencing E-LTP fail to capture new PRPs 2 hours after the induction of LTP (Frey and Morris, 1998b). There is then, a limited time window for the overlap between an receptive synapse and the arrival of PRPs outside of which, these two elements will fail to interact and the synapse reverts to pre-stimulation strengths.

Figure 11.7 The 'receptive synapse' model of the STC hypothesis.

Heterosynaptic plasticity behind the STC hypothesis understood through the 'receptive synapse' model.

Weak tetanization capable of inducing protein synthesis independent E-LTP fails to maintain changes in expression (upper panel). This short-lasting LTP is capable of unlocking the synapse (tag) but not to induce the synthesis of PRPs. As time passes, the mechanisms of expression do not find the stabilizing support necessary to:

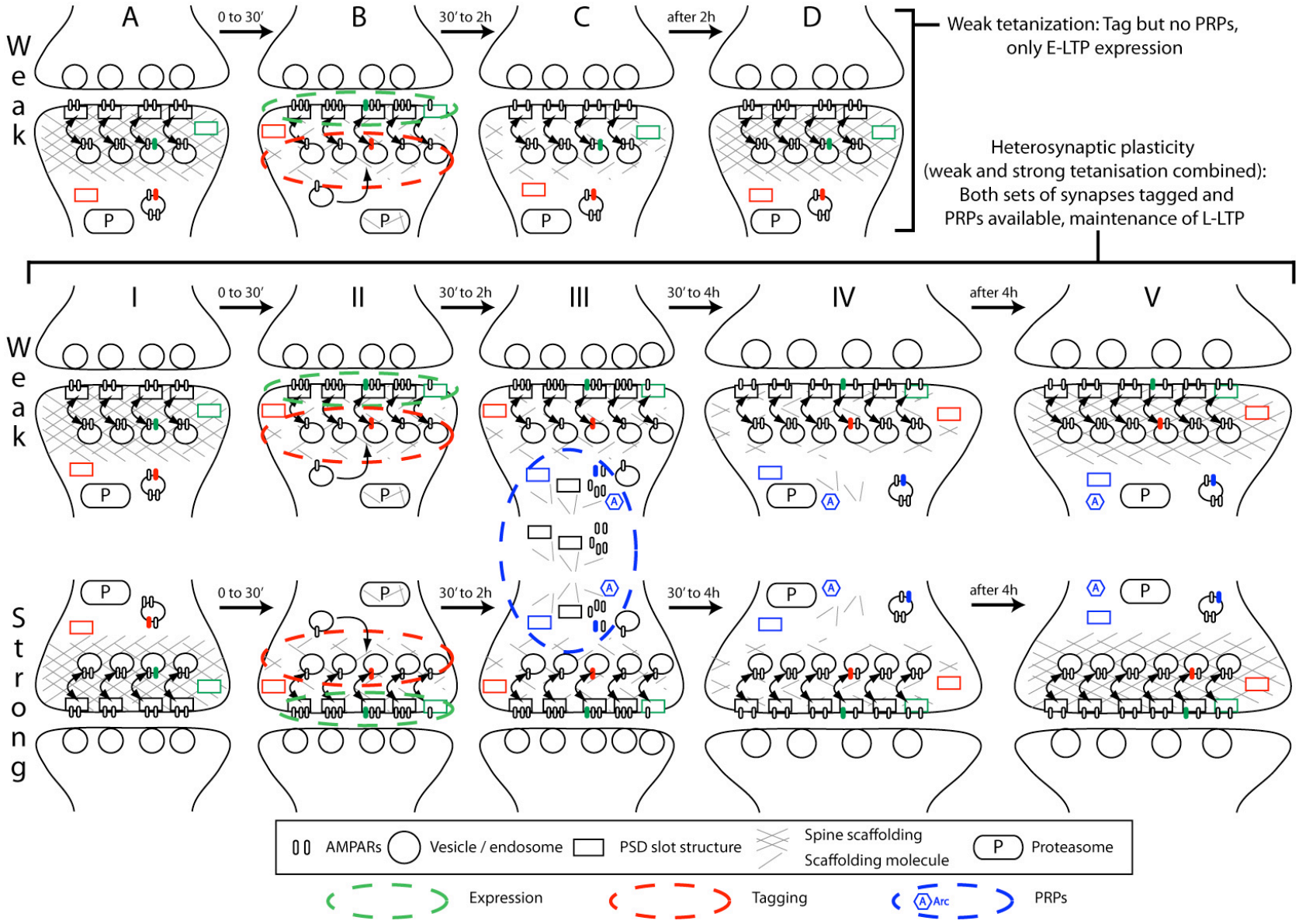
a) **Support the addition of new 'slots' for the incorporation of receptors. Without the PRPs, the number of scaffolding molecules driven into the PSD in the early phases of LTP (expression) will decrease due to the standard molecular turnover of the synapse. Without the addition of scaffolding molecules to a reserve pool in the spine, the recycling of receptors will eventually bring the size of the synapse back to a pre-tetanization state.**

b) **Prevent the return to prestimulation levels of AMPAR slots due to molecular turnover of AMPAR subunits. This is a consequence of point (a).**

c) **Build the presynaptic machinery responsible for the increase in vesicle release.**

d) **Expand the spine through actin conformational changes.**

However, when the weak tetanization is combined with the strong (L-LTP inducing) stimulation to another convergent set of synapses (lower panels), the PRPs synthesized in response to the strong input are available to the unlocked synapses of the weak pathway. The combination of an receptive (tagged) synapse and PRPs allows for additional changes in the synapse and the stabilization of those synaptic changes (Fig. 11.6).


A
0 to 30′
B
30′ to 2h
C
after 2h
D
Weak
P
Weak tetanization: Tag but no PRPs,
only E-LTP expression
Heterosynaptic plasticity
(weak and strong tetanisation combined):
Both sets of synapses tagged and
PRPs available, maintenance of L-LTP
I
0 to 30′
II
30′ to 2h
III
30′ to 4h
IV
after 4h
V
Strong
A
AMPARs
Vesicle / endosome
PSD slot structure
Spine scaffolding
Scaffolding molecule
Proteasome
Expression
Tagging
Arc
PRPs

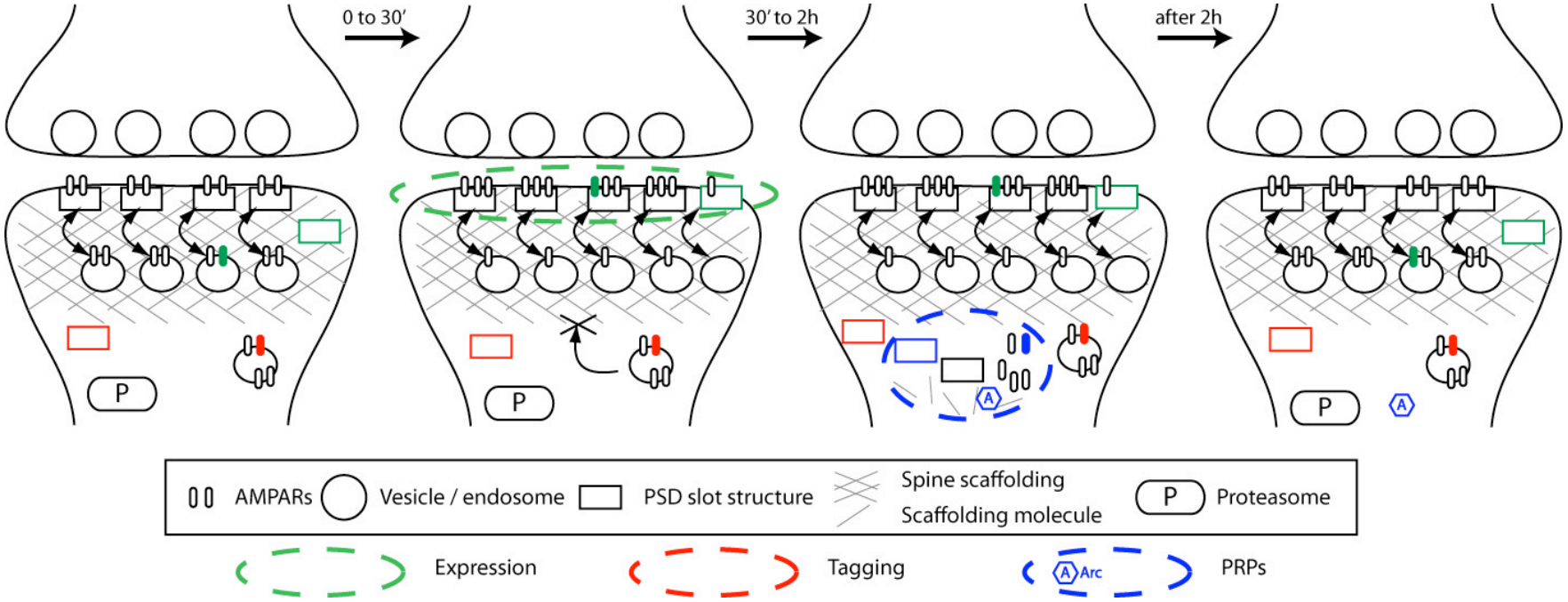
11.4.6 CaMKII block through KN-93 under the 'receptive synapse' model

After explaining the 'receptive synapse' model, we can go back to some of the experiments reported in this thesis. The low dose KN-93 experiments revealed a way of blocking the setting of the tag without affecting the expression of LTP or the synthesis of PRPs (Chapter 6). The data shows normal expression of LTP for a few hours before it starts decaying to baseline levels. At the same time, the tag has been blocked. This dissociation between expression mechanism and the setting of the tag fits the 'locked synapse' model. While calcium entry at the time of induction is capable of engaging the processes responsible for the incorporation of AMPARs into the PSD, the unlocking of the scaffolding holding the spine together is compromised by KN-93. Recently, the incorporation of the AMPAR subunit GluR1 has been shown to be necessary but not sufficient for the expansion of the synapse. Chemically induced LTP expression and addition of AMPARs was independent of spine expansion, which required NMDAR activation (Kopec et al., 2007), and according to our results, downstream activation of CaMKII (chapter 6). It seems then that under 1 μM KN-93, the PSD remains locked and the number of receptor 'slots' is not increased even after the arrival of new PRPs. With time, the mechanisms responsible for the expression of LTP fail to find the structural support needed for their stabilization and the synapse reverts to pretetanization strengths (Fig. 11.8). There is recent evidence of the need of CaMKII to stabilize the expansion of the spine through the stabilization of changes in the actin cytoskeleton (Honkura et al., 2008).

Figure 11.8 Sequence of events when the tag process is blocked.

The block of L-LTP with the low dose KN-93 (1 μM) can be understood within the framework of the 'receptive synapse' model as the result of the inability of the synapse to unlock its PSD. While the mechanisms of early expression do still potentiate the synaptic response after strong tetanization, the inhibition of CaMKII by the KN-93 drug prevents the loosening up of the PSD, the expansion of the actin cytoskeleton, and the incorporation of PRPs whenever they become available.

The E-LTP expression mechanisms are not supported by morphological changes in the PSD or in the presynaptic side of the synapse and the synaptic strength, through normal molecular turnover, returns to a prestimulation strength.


0 to 30′
30′ to 2h
after 2h
P
P
P
A
AMPARs
Vesicle / endosome
PSD slot structure
Spine scaffolding
Scaffolding molecule
P
Proteasome
Expression
Tagging
A Arc
PRPs

11.4.7 Competitive maintenance explained by this model

Fonseca et al., 2004 describes "competitive maintenance" as the phenomenon by which under regimes of reduced protein synthesis, induction of additional potentiation on one set of synapses leads to depotentiation of another (Fonseca et al., 2004). This phenomenon is restricted to synapses within the same dendritic compartment and is modulated by the strength of the stimulation (i.e. the stronger the stimulation, the bigger the depotentiation on the independent pathway). They also show that this effect is specific to potentiated pathways and does not occur in a control, unstimulated pathway when another is reactivated under the effects of anisomycin. This is an important piece of the puzzle around the STC hypothesis since it shows that for a pathway to experience depotentiation under a regime of reduced protein synthesis, it has to have experienced previous potentiation. Under the locked synapse model, only receptive synapses trying to stabilize their new conformation require new gene products (PRPs). Two sets of synapses with receptive PSDs will tap onto common pool of PRPs under a regime where those PRPs are scarce. The further unlocking and potentiation of one set of synapses will draw diffusible PRPs out of the pool. As a consequence of the decrease in the pool of PRPs, the dynamic turnover on the other receptive set of synapses will result in a reduction in the number of slots and the subsequent depotentiation observed experimentally.

Evidence supporting this view comes from the recruitment of new Homer1a protein from the dendrite into the synaptic spine after synaptic activation (Okada et al., 2009). The redistribution of the protein through passive diffusion around the area of stimulation (dendritic compartment) may draw a scarce Homer (PRP) away from unstimulated synapses. A locked synapse, on the other hand, will be immune to the redistribution of scarce PRPs.

11.4.8 LTD and cross-tagging in this model

In the case of LTD, STC experiments provide ain interesting clue about the mechanisms behind synaptic plasticity. Even though the direction of change in

synaptic strength in LTD is the opposite of that of LTP, their maintenance mechanisms share enough properties to allow for 'cross-tagging' (Sajikumar et al., 2007). In a cross-tagging stimulation protocol, a weak tetanus capable of producing E-LTP is paired with strong stimulation capable of inducing L-LTD. Interestingly, the E-LTP is rescued into L-LTP and this is interpreted as evidence for a shared, or at least overlapping, pool of PRPs between the two types of changes in long-term plasticity. The tags, on the other hand, have different induction properties as CaMKII is necessary for the setting of the LTP tag (chapter 6) but not for the setting of the LTD tag (Sajikumar et al., 2007). The 'receptive synapse' model of STC described here accounts for cross-tagging in heterosynaptic plasticity. In the case of LTD, the PRPs act in the same way as in LTP, supporting and maintaining the change in synaptic strength. The difference is the size of the PSD that needs to be locked or stabilized (i.e. strengthened in LTP and reduced in LTD). In other words, expression of LTP or LTD is parallel to a common unlocking of the PSD (tag), necessary for the maintenance of whichever change has taken place.

How is LTD different than LTP? While the expression of LTP involves the phosphorylation of Ser-831 of the GluR1 subunit of the AMPAR, the expression of LTD dephosphorylates Ser-845 (Lee et al., 2000; Kemp and Bashir, 2001). This difference results from the different kinases and phosphatases activated (CaMKII for LTP, PKA for dedepression and PP1 for LTD and depotentiation). In LTD, contrary to LTP, the dephosphorylation of Ser-845 by PP1 moves AMPAR out of the PSD, and the slots available to AMPAR are reduced. It is known that Arc/Arg3.1 binds the endocytic proteins dynamin-2 and endophilin-3, forming a complex that regulates the endocytic trafficking of AMPA-type glutamate receptors (Shepherd et al., 2006). Lack of Arc leaves AMPAR in the membrane surface (Chowdhury et al., 2006) and translation of Arc protein brings down the number of AMPARs in the PSD via a calcineurin dependent mechanism (Rial Verde et al., 2006). Arc is a PRP translated after NMDAR as well as PKA activation (Bloomer et al., 2008). Some models suggest that Arc function may be to down-regulate AMPAR expression at non-potentiated synapses (Rial Verde et al., 2006; Abraham and Williams, 2008) but Arc could as well be responsible for immediate expression of LTD and new Arc protein may be necessary for the maintenance of that depotentiated state. There is some

controversy about the role of Arc in LTP, if any, after studies using Arc anti-sense mRNA to block LTP (Guzowski et al., 2000; Messaoudi et al., 2007).

Although the expression of LTD is accounted for by different mechanisms than that of LTP, the signalling pathways necessary for the synthesis of PRPs after LTD induction may rely on the same kinases as LTP (Ras and MAP Kinase and CaMKIV). But if CaMKII is not activated after LTD is induced, what molecule is responsible for the unlocking (tagging) of the depressed synapse? A candidate for an LTD tag molecule is calcineurin, PP2B, a phosphatase necessary for the destabilization of the actin cytoskeleton after NMDAR activation (Halpain et al., 1998). LTD would reach a state of destabilized F-actin (Okamoto et al., 2004) permissive for the capture of PRPs (i.e. additional Arc) and the change in spine structure (i.e. reduction of PSD hyperslots for AMPARs). This means that as with LTP, the mechanisms of expression of LTD may be dissociated from the unlocking of the scaffolding and the tagging of the synapse allowing for actin changes without expression of synaptic depression and viceversa (Okamoto et al., 2004). Indeed, LTD is associated with the shrinkage of the synaptic spine that is dependent on the phosphatase calcineurin. Similarly to what can be seen in LTP, in LTD there is also a dissociation between expression, that requires calcineurin-dependent PP1 activity, and the pathway leading to the shrinkage of the spine which relies instead on calcineurin-dependent cofilin-mediated depolymerization of the actin cytoskeleton without the need for PP1 (Bamburg, 1999; Zhou et al., 2004; Bastrikova et al., 2008).

So, similarly to what happens during LTP, in LTD we find specific mechanisms of expression (i.e. calcineurin activation of PP1), the need for new PRPs required to stabilize the synaptic change (i.e. Arc protein) and the unlocking of the synapse (i.e. calcineurin activation of cofilin). Both LTP and LTD would depend on the unlocking of the synapse as the enabling mechanism for the capture of specific molecules from a shared pool of PRPs.

Across a particular neuron, new information would be encoded by potentiating and depressing synapses. This would require the upregulation of LTD-PRPs even after LTP induction and after every learning episode (Miyashita et al., 2009). Indeed, the phenomenon of ‘cross-tagging’ reveals that gene products are shared between synapses expressing LTP and LTD (Sajikumar and Frey, 2004a). The contents of the

pool of PRPs may be determined by the interaction between activated kinases as determined by different profiles of NMDAR activation (Coba et al., 2008). The need for LTD-specific proteins can be signalled via calcineurin, as it controls transcription by activating CREB-dependent genes while inhibiting SRE-dependent transcription (Lam et al., 2009). In this way, calcineurin could influence the balance between LTP-PRPs and LTD-PRPs.

Once the synapse is allowed to change conformation (unlocked), the direction of this change may be influenced by the insertion of lipid membrane during LTP or the endocytosis seen in LTD. While the unlocking would be a common feature of both LTP and LTD, the ultimate change in synaptic weight would be determined by the specific molecules responsible for its expression and by the later addition of also specific PRPs. PSD-95, for instance, has been shown to have opposite effects on LTP than another scaffolding molecule PSD-93 (Carlisle et al., 2008a). Also, since LTD relies on Arc for the endocytosis of AMPARs (Chowdhury et al., 2006) and the presence of the GluR1 subunit of the AMPAR in the PSD is necessary to maintain its structure (Kopec et al., 2007), the removal of AMPAR from the surface may also be sufficient to reduce the size of the dendritic spine (Shepherd et al., 2006).

To summarize, the 'receptive synapse' model of STC attempts to explain LTP and LTD maintenance by proposing that the local changes responsible for synaptic plasticity can be further divided in two processes. First, the mechanisms of depression or potentiation of the synaptic response particular to LTP and LTD. Second, a shared process of untangling or unlocking of the scaffolding and support structures in the spine such that the new state of expression can later be stabilized thanks to the contribution of PRPs. These PRPs will stabilize opposite states; (i) in LTP, the unlocking of the synapse is necessary for the expansion of the PSD (or addition of Lisman's hyperslots) and for the subsequent stabilization of that expansion; (ii) in LTD, the unlocking of the PSD is necessary for the PRPs to stabilize the shrinkage of the PSD (or removal of hyperslots).

In this way, the 'receptive synapse' model of STC explains the dissociation between the expression of synaptic plasticity and the setting of a tag. Without the tag (the unlocking), expression of LTP and LTD can still take place temporarily. Without the tag, however, in the case of LTP the additional AMPARs or the new

presynaptic vesicles fail to secure and stabilize their position due to the lack of support structures. In the case of LTD, without the tag, the removal of AMPARs leaves behind their support structure (scaffolding and PSD slots), so after the effects of early expression (phosphatase activity) wear off, the molecular turnover at the synapse will replenish those empty slots and the synapse reverts to baseline levels of synaptic strength.

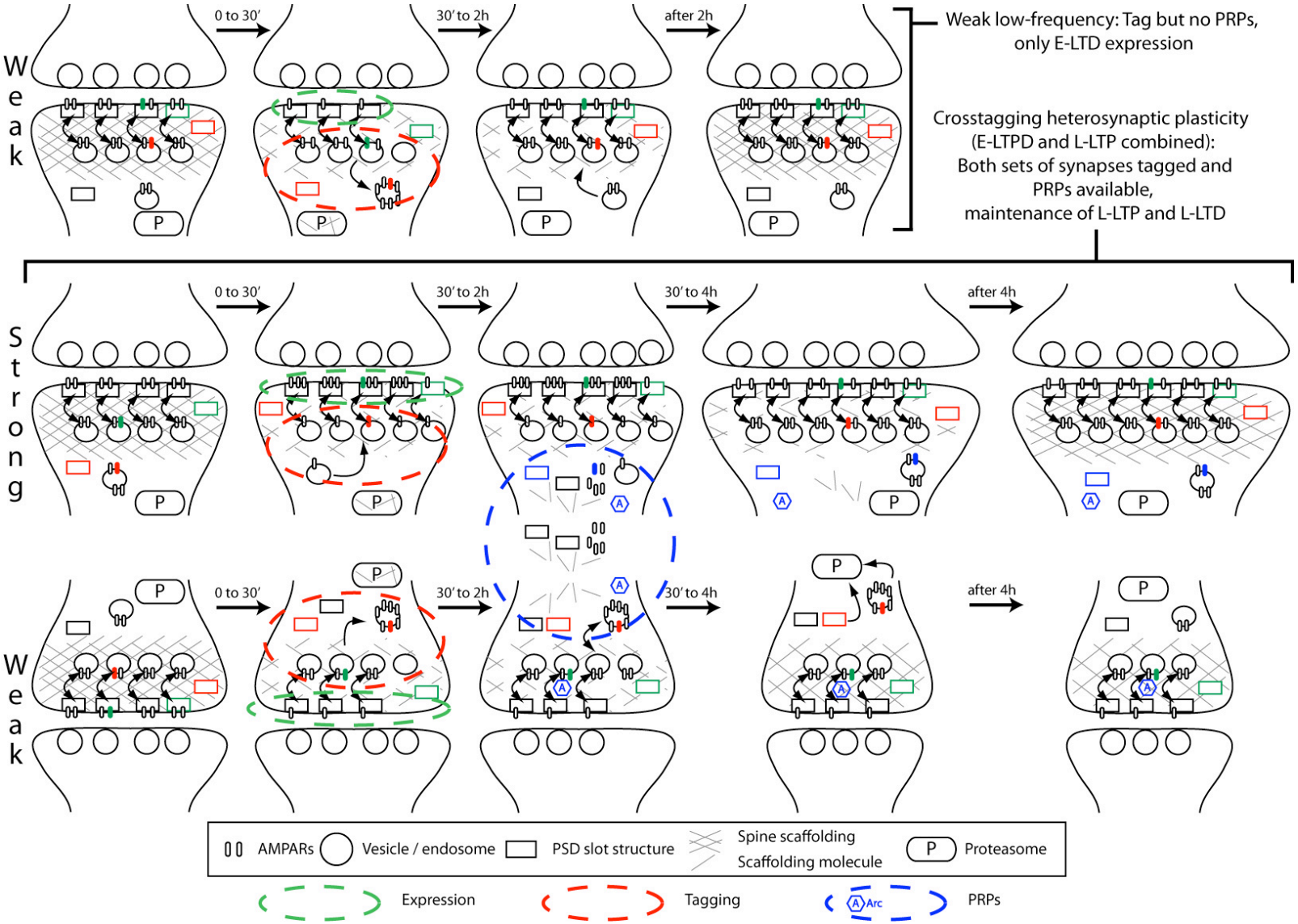
As informative as the cross-tagging experiments may be to understand the interactions between tags and PRPs, what do they tell us about how a particular neuron encodes a new configuration of synaptic weights on its synapses? The induction protocols used to elicit LTP and LTD are quite artificial in the sense that a particular neuron will probably receive continuous inputs that may require the potentiation of some synapses and the depression of others. The information carried by a signal strong enough to engage into the synthesis of PRPs (and synaptic learning), will probably be represented as a combination of both increases and decreases in efficacy at some synapses within the same neuron. It makes sense then, to expect that some synapses will need to be stabilized in a depressed state while others will have potentiated their synaptic strength. The receptive synapse model proposes the local synaptic control of expression (LTP or LTD) combined with an unlocking of the spine (tag) that makes the synapse receptive to PRPs capable of stabilizing the local change. Independently of the direction of the synaptic change, locked synapses will not have access to PRPs while unlocked synapses that do not receive PRPs will eventually revert to a prestimulation state.

Figure 11.9 The ‘receptive synapse’ model of cross-tagging.

The phenomenon of cross-tagging as explained by the ‘receptive synapse’ model.

Upper panel: Stages after the induction of E-LTD. First, the expression of LTD involves the shift of AMPAR turnover towards endocytosis and away from insertion into the PSD. With weak low-frequency stimulation there is expression of LTD and unlocking of the synapse (tag) as described above. However, there is no synthesis of PRPs. Without additional PRPs, the synapse cannot be locked in its depotentiated state and the molecular turnover allows the scaffolding molecules to return to their pre-depression levels. AMPAR subunits will be dynamically redistributed to baseline levels.

During cross-tagging, however, the LTP induced in one set of synapses shares PRPs from a common pool from which LTD expressing synapses can also benefit. The arrival of new PRPs (Arc, PSD-93,…) can now sustain the reduction in synaptic strength and the synapse is locked in its depressed state.


Weak
0 to 30′
30′ to 2h
after 2h
P
Weak low-frequency: Tag but no PRPs,
only E-LTD expression
Crosstagging heterosynaptic plasticity
(E-LTPD and L-LTP combined):
Both sets of synapses tagged and
PRPs available,
maintenance of L-LTP and L-LTD
Strong
0 to 30′
30′ to 2h
30′ to 4h
after 4h
A
Weak
0 to 30′
30′ to 2h
30′ to 4h
after 4h
AMPARs
Vesicle / endosome
PSD slot structure
Spine scaffolding
Scaffolding molecule
Proteasome
Expression
Tagging
Arc
PRPs

11.4.9 Resetting of the tag explained by the 'receptive synapse' model

Another mechanism responsible for the type of heterosynaptic plasticity explained by the STC hypothesis is revealed by the low-frequency (LFS) depotentiation of L-LTP (Bashir and Collingridge, 1994; Staubli and Chun, 1996; Sajikumar and Frey, 2004b; Young and Nguyen, 2005; Young et al., 2006). This LFS acts on the tag and not on the availability of PRPs (Sajikumar and Frey, 2004b; Young and Nguyen, 2005). Low-frequency stimulation engages phosphatases that block the expression of LTP and the expansion of the synapse (Woo et al., 2002; Yang et al., 2008). Indeed, this LTP-tag-blocking LFS requires the actions of PP1 and is associated with a reduced activity of PKA (Young et al., 2006). Maybe an active PP1 at the synapse could block or impair the activation of CaMKII during the induction of LTP, consequently blocking the tag. Interestingly, the time window for the effect of the LFS is restricted to 5 min after the 'tagging' stimulation. This is, if the LFS is delivered 10 min after weak tetanization of one pathway, the gene products of a strong tetanization of an independent pathway are captured by the weakly stimulated set of synapses and L-LTP is maintained (Sajikumar and Frey, 2004b). One clue to explain this phenomenon comes from the fact that L-LTP is expressed even after the full depotentiation caused by the LFS. How could one set of synapses that had reverted to baseline levels of strength after LFS, re-potentiate and express LTP? The recovery of potentiations offer further evidence for a dissociation between the mechanisms of expression of LTP (i.e. AMPAR number or properties, presynaptic vesicle number or release probability) and the mechanisms responsible for the maintenance of synaptic weights (shape of PSD, number of PSD puncta, shape and conformation of the scaffolding proteins supporting slots (full or empty)). Because phosphatases are responsible for the resetting of the tag after LFS (Woo et al., 2002; Young et al., 2006), they would be expected to affect both the expression of synaptic change (depotentiation through AMPAR dephosphorylation) as well as the unlocking of the molecular scaffold through their inhibition of the necessary CaMKII. Indeed, independently of when LFS is delivered, the expression of LTP is always blocked (maybe by action of phosphatases on AMPARs surface expression). However, if the

actions of the LFS-driven phosphatases arrive too late (after 10 min), the PSD is already unlocked (maybe by CaMKII) and new scaffolding slots have been added. These slots will still require new PRPs later but if they are made available by de novo PRP synthesis, AMPARs will fill them, LTP will be successfully re-expressed and maintained and the initial depotentiation of the LFS forgotten.

11.4.10 Slow-onset plasticity.

The 'receptive synapse' model of STC can also explain why certain chemicals (dopamine, BDNF, forskolin, …) are capable of triggering a delayed synaptic change.

BDNF triggers L-LTP in the hippocampus but it does so in a delayed form that does not require NMDAR activation (Messaoudi et al., 2002). BDNF application on hippocampal slices upregulates gene transcription and translation (Liao et al., 2007). This is probably mediated by ERK and other kinases (Hetman et al., 1999) and the end result is synthesis of PRPs. In addition to this, BDNF promotes cytoskeletal changes in the hippocampus by expanding the actin cytoskeleton (Rex et al., 2007). BDNF induces dendritogenesis through local calcium influx and this effect is blocked by KN-93, suggesting a BDNF-dependent activation of CaM Kinases as the mechanism downstream of BDNF responsible for the tagging of the synapse (Takemoto-Kimura et al., 2007). The observed result of these two actions, PRP synthesis and cytoskeletal changes, support the necessary role of BDNF in LTP (Bekinschtein et al., 2007). Now, the 'receptive synapse' model of STC can explain why the actions of BDNF application produce the delayed NMDAR independent potentiation. While on one side BDNF upregulates the synthesis of PRPs, its actions on the cytoskeleton expand the scaffold holding together the synapse and the PSD, making room for new PRPs and effectively tagging all synapses. The unlocked synapses accept the PRPs and build the scaffold that is slowly filled by AMPARs on the postsynaptic side and vesicles on the presynaptic side. Similar results to those of BDNF application are seen with neurotrophin-3 (Kang and Schuman, 1996). More recently, this 'BDNF-dependent tagging' has been confirmed to be independent of immediate expression of LTP and to be capable and necessary to enlarge dendritic spines (Tanaka et al., 2008).

The case of slow-onset plasticity due to dopamine application is more puzzling as while high (50 μM) levels of dopamine induce a delayed LTP low levels of dopamine (10 μM) induce a delayed LTD (Sajikumar and Frey, 2004a). The delayed LTP can be explained in a manner slightly different to that of BDNF, this is, through an NMDAR dependent unlocking of the synapse by the test stimulation coupled with the synthesis of PRPs by the dopamine agonist (Navakkode et al., 2007). It is harder to explain, however, the effect of moderately increased activity of PKA (after application of 10 μM of dopamine) leading to LTD. Maybe the explanation resides on the different actions of low levels of PKA at the synapse, since dopamine agonists on their own can induce LTD (Chen et al., 1995). Further evidence that chemical LTP is capable of setting tags and inducing the synthesis of PRPs without an immediate effect on the expression of synaptic changes comes from the effect of LFS on forskolin induced LTP (Young et al., 2006). LFS is known to specifically block tag setting without interfering with the pathways leading to the synthesis of PRPs (see previous section (Young and Nguyen, 2005)). Pairing of LFS with forskolin-induced chemical LTP also impairs the maintenance of L-LTP and reveals controversial role of PKA in the setting of the LTP-tag (Young et al., 2006).

The slow and incremental potentiation described in this section has also been reported when replaying certain place firing patterns (Isaac et al., 2009). In this case, the pairing of synaptic inputs with back-propagating action potentials may be capable of tagging synapses for LTP while the bp-AP on their own, upregulate PRP synthesis.

To summarize, the 'receptive synapse' model distinguishes between tag-setting and the expression of synaptic changes acting locally at the synapse while de novo PRPs are required for the maintenance of the change. The model, with the three elements (two local and one cell-wide), explains slow-onset plasticity as an interaction between tag-setting and PRP synthesis which is independent of the initial expression of plasticity. The receptive synapse (tagged) slowly incorporates PRPs and then starts expressing LTP (or LTD).

11.4.11 Summary of the 'receptive synapse' model

To summarize, the locked synapse model of synaptic plasticity proposes the dissociation between the expression of synaptic plasticity and the processes involved in the tagging of the synapse. This dissociation was not present in the original STC hypothesis (Frey and Morris, 1998a) and is based on the work presented in this thesis as well as recent findings reported by others. The predictions of the model are based on an extensive body of literature dealing with the roles for kinases, receptors and scaffold molecules during the early and late stages of synaptic changes, both pre and postsynaptic. All this information is brought together by the research on one particular form of heterosynaptic plasticity, explained by the STC hypothesis. The experiments undertaken in this thesis (Chapter 3 to 7) were critical in advancing the understanding of the processes behind this form of heterosynaptic plasticity. In chapters 3 to 5, the replication of STC experiments with low-frequency test stimulation allows for very long lasting recordings. One first clue that leads to the receptive synapse model is the distinct decay rates in the expression of LTP depending on the frequency of test stimulation. This suggested the activity-dependent use of PRPs already confirmed by others (Fonseca et al., 2004; Fonseca et al., 2006a). On top of this, experiments in chapters 6 reveal a way of blocking the tag without interfering with the availability of PRPs or with the early expression of LTP. In chapter 7, the opposite occurs, a drug is used that blocks the synthesis of PRPs while not impairing the setting of the tag. Benefiting from the STC hypothesis, the receptive synapse model tries to bring these results, and others from the literature, together in an attempt to understand the processes involved in synaptic plasticity.

As discussed in chapter 1, the STC hypothesis, through its understanding of synaptic plasticity, may provide clues as to the requirements for the maintenance of memories. Part of the work in this thesis tried to assess the relevance of the STC hypothesis in memory maintenance and the following section attempts to clarify this role.

11.5 Memory modulation explained by the STC hypothesis

Following the synaptic plasticity and memory hypothesis, one may predict that in the same way that weakly induced synaptic plasticity benefits from the availability of PRPs, a weakly encoded memory with a short retention time may lengthen its maintenance if PRPs are made available to the synapses encoding it. With this in mind, the work of this thesis moved towards behavioural protocols capable of testing this hypothesis.

In the experiments described in chapters 8 to 10, the goal was to modulate the persistence of a weakly encoded, short-lasting memory so as to be present for a longer time. Using the STC hypothesis, the enhancement of memory was approached from a molecular framework rather than from the systems level, where most of the literature comes from (McGaugh, 2000). I assumed that the same processes capable of sustaining changes in synaptic strength would be required to maintain a spatial memory and, in particular, that the interaction between synapse specific and cell wide processes would allow for memory consolidation. However, instead of a bottom-up approach, most research done in memory consolidation revolves around system interactions between major areas of the brain and their hormonal or neurotransmitter inputs. This is, memory retention can been lengthened by behavioural and drug manipulations, while, independently, LTP maintenance can also be artificially modulated. The behavioural tagging experiments described in this thesis are an attempt to use the knowledge developed at one reductionist and molecular level to learn about a higher and holistic memory mechanism. In achieving this, we hoped that some light could be shed into molecular mechanisms behind the rich literature dealing with memory modulation.

In chapter 10 we approach the challenge of translating the STC hypothesis into a behavioural correlate by making use of a delayed match to place (DMP) protocol in the water maze. In this type of task the encoding of a platform location changes every day and because of new memories being constantly formed, the strength of the encoding can be lowered up to a point where the persistence of the memory is limited to 6 or 24 h. Once a weak encoding protocol is at hand, the goal was to use some sort

of behavioural manipulation to enhance the maintenance of the memory. After that, we would have to confirm that the mechanism of improvement is related to the processes responsible for the heterosynaptic plasticity seen in STC experiments. In the watermaze experiments, we decided to use cold-water stress to enhance memory maintenance but as described in Chapter 10, the bell curve of the effects of stress probably was responsible for the impairment, not the enhancement, in memory performance that we obtained. This impairment in the memory for the correct location was already detected after a short retention delay, suggesting that maybe the treatment with the acute cold-swim stress before the encoding had an effect in the encoding, in addition or parallel to any effect on memory maintenance. In any case, the water maze DMP task and the cold water stress did prove to be steps in the wrong direction and a different approach was developed to tackle the 'behavioural tagging' project (chapters 8 and 9).

In chapter 8, we run similar delayed match to place experiments in a dry maze, the event arena. One of the advantages of this setup is that the additional behavioural manipulation responsible for a potential enhancement in memory retention could be presented in the same environment as where the encoding of the memory takes place. This was relevant for the planning of the experiment since the translation of the STC model into behaviour predicts that the synthesis of PRPs, by the behavioural manipulation, should to take place in the same cells encoding the memory. In the event arena, we found that the exploration of a novel environment, sometime before the encoding of a memory that decays within 6 hours, improved the retention at that 6-hour time point as to allowing the animals to perform above chance levels. This was encouraging but subsequent experiments trying to pinpoint the mechanisms responsible for this effect run into performance problems and high variability between individuals. The question remained as to whether the effect detected in this set of experiments was related to the mechanism responsible for the heterosynaptic plasticity behind the STC hypothesis.

In chapter 9, we modified and simplified the dry maze DMP task in order to sustain the performance of a group of animals through multiple tests and drug manipulations. We succeeded in replicating the results reported in chapter 9 and we confirmed the necessary role of protein synthesis, and D1/D5 receptors in the

enhancement of the place-memory retention observed after the exploration of a novel environment. With the appropriate controls, we confirmed that we have a spatial task that manipulates memory retention through similar mechanism as those that are responsible for the synthesis of PRPs in the hippocampus. It is tempting to speculate that this is a behavioural correlate of the STC hypothesis (i.e. behavioural tagging), similar to the effects reported previously in non-spatial tasks (Moncada and Viola, 2007; Ballarini et al., 2009).

11.6 Future research inspired by these results

The electrophysiological results presented in this thesis (chapters 3 to 7) make use of the theoretical framework behind the synaptic tagging and capture hypothesis to test the roles of distinct CaM Kinase pathways in the maintenance of LTP. The tools employed to explore those roles (i.e. the use of tag-block and prp-block experiments) should not be limited to these particular set of kinases. The strategies can be reapplied to different target molecules as specific inhibitors become available. I will mention some candidates for subsequent experiments below.

Behaviourally, chapters 8 to 10 describe attempts to develop models capable of testing and building on top of the findings that the electrophysiology of heterosynaptic plasticity has revealed. We now have a behavioural task capable of modelling and questioning hypotheses that until now have been mostly restricted to synaptic plasticity as seen *in vitro*. As more is discovered about the mechanisms behind the STC hypothesis, we hope to be able to use the behavioural tool described in chapters 8 and 9 to probe the behavioural significance of molecular approaches to synaptic plasticity. It would be interesting to connect the experiments reported in chapters 6 and 7 to the behavioural tagging tasks. However, one has to keep in mind the limited accessibility of the intact brain to drug manipulations.

The first type of experiment that one could attempt would be the more straightforward use of STO-609 in behavioural tagging. The experiment would mimic the protocols used to identify the actions of protein synthesis inhibitor anisomycin and the D1/D5R blocker SCH23390. First, one would show that STO-

609 has the ability to block the long-term memory induced by a 'Strong' encoding protocol (3-pellets). Then, the novel exploration would be presented before the STO-609 block and the hypothesis sustaining a specific role of CaMKK in the availability of PRPs would predict a rescue of the 3-pellet memory.

The use of KN-93 and other CaMKII inhibitors in STC may prove much more complex due to the concentration dependent effects of those drugs. The use of other CaMKII inhibitory peptides available at this time (i.e. AIP) carries the problem of permeability. These peptides are usually attached to permeabilizing motifs but, unfortunately, these motifs have their own effects on plasticity that prove difficult to control. Once the appropriate CaMKII inhibitor has been developed, it is only logical to attempt a tag-block experiment in the behavioural task described in chapters 8 and 9. This would become an attempt to run a 'tag-blocking' experiment in a behavioural task and would consist of a weak encoding protocol followed by the encoding of a strong memory under the influence of the CaMKII inhibitor. My hypothesis would expect the weak memory to be present after 24 hours while the strong memory would have decayed. That result would mimic the cross-over of LTP expression observed with 1 μM KN-93 and described in Chapter 6.

Even with the right biochemical tools, however, there is still the problem of having to determine when a strong memory has been blocked by a CaMKII inhibitor. In the protocol used in this thesis we have monitored the exploratory behaviour of the rats after a second exposure to the novel box (24 h after their first encounter with it). The only evidence that we have for a long-lasting (24 h) memory for the novel box is based on the decreased path length walked by the animals in the 5 minute exploration (Figure S6 in Appendix). Because a claim for the block of a strong memory by a CaMKII inhibitor would require a more robust measure of memory, an initial step towards a behavioural tag-block experiment could consist of the development of a strong encoding protocol that can be paired with the weak memory without causing interference. This may require the use of two event arenas with an independent memory for a rewarded location being encoded in each room.

On the electrophysiology of LTP, the results presented in this thesis have validated a methodological protocol capable of dissociating roles for molecules necessary for the setting of local synaptic tags or necessary for the availability of PRPs (i.e. the

pairing of weak and strong stimulation protocols with drugs present during one or the other). The tools developed and used in chapters 3 to 7 could be used to learn more about the many signalling molecules involved in synaptic plasticity. There are other candidates for tag-specific roles and it will be a goal for the future to directly test them. For example, Myosin Vb is required for LTP (Wang et al., 2008) by contributing to the exocytosis of lipid membrane AND AMPARs necessary for LTP. This is a potential tag that can be put to the 'tag block' test. Another Myosin (II) can be targeted with Blebbistatin, and has already been shown to both impair LTP and long-term memory (Miller and Rumbaugh, in prep.). Contrary to the problems of infusing KN compounds into the brain, the use of Blebbistatin should not suffer from too many concentration-dependent effects. If the block of myosin II is capable of impairing long-term memory, and the necessary actions of myosin II in synaptic plasticity are restricted to local synaptic effects (i.e. control of exocytosis and AMPAR incorporation), we can envisage using Blebbistatin in a behavioural tagging protocol. First, the electrophysiology has to confirm that the effects of Blebbistatin are capable of, and restricted to, blocking of the synaptic tag without an effect on the availability of PRPs (i.e. test for tag-block). If that is the case, Blebbistatin can then be used in a behavioural tagging experiment in which the drug is present during a 'strong' encoding trial and blocks the long-term memory for that event. The interesting question will come from the pairing of a 'weak' encoding protocol, without any drug infusion, with the inhibited 'strong' encoding by the actions of Blebbistatin. A hypothesis working within the STC framework would predict that the weak memory, that usually decays before 24 h, will now be sustained for longer.

Overall, I see the STC hypothesis as a valuable framework through which to learn about synaptic plasticity. The value of that research, however, will be limited if it devolves into a reductionist race to find every single molecule involved in synaptic plasticity. On the other hand, the successful translation of electrophysiological findings into behaviour, by using tasks like the one-trial match to place in the event arena, will bring us much more interesting information. It is by connecting the molecular knowledge to behavioural performance that neurobiology can reinforce psychology to the benefit of all.

A fuller understanding of the intricacies of synaptic plasticity will not only allow for the basic understanding of the brain and the mind, but will certainly evolve into clinical and medical applications to the betterment of humankind. As Francis Bacon wrote in *Meditationes Sacrae* (1595) "*Scientia potentia est*": Knowledge is power.

Appendix 1: Supplemental data

Chapters 6 and 7

Methodology concerning hippocampal dissociated cultures

Hippocampal dissociated cultures were prepared from neonatal Wistar rats and cultured on coverslips as described previously (Bito et al., 1996; Kawashima et al., 2009). At 20-21 days *in vitro* (div), at which synaptic network in the culture was well developed, neurons were silenced with 2 μM TTX for 2 h and treated with various concentrations of kinase inhibitors (KN-93 or STO-609 in 0.1 % DMSO) for 30 min in Tyrode's solution [NaCl 124 mM, KCl 2.5 mM, NaH_2PO_4 1.0 mM, $CaCl_2$ 2.0 mM, $MgCl_2$ 2.0 mM, $NaHCO_3$ 24.6 mM, D-glucose 30 mM, HEPES (pH7.4) 20mM]. The cultures were then stimulated with 10 μM Glutamate/100 μM Glycine/1 μM TTX in 0 Mg^{2+} Tyrode's solution for 3 min in the presence of the inhibitors. After stimulation, neurons were immediately fixed in chilled methanol for 5 min followed by ice-cold 4% paraformaldehyde/4 % sucrose/phosphate-buffered saline (PBS) for 5 min.

Immunocytochemistry was performed as essentially described previously (Kawashima et al., 2009). Briefly, the fixed cells were washed, permeabilized, and incubated in a blocking solution (3% BSA/0.3% Triton X-100/PBS) with a phosphatase inhibitor cocktail (PhosSTOP, Roche). The cells were then reacted with primary antibodies in the blocking solution. The primary antibodies used were anti-phosphoCREB (rabbit mAb, Epitomics) and anti-MAP2 (mouse mAb, Sigma), or anti-phosphoCaMKII (rabbit pAb, Promega) and anti-CaMKIIα (mouse mAb, Invitrogen). After the wash, the primary antibodies were labeled with anti-mouse AlexaFluor488- and anti-rabbit AlexaFluor594-conjugated secondary antibodies. The cells were then washed, stained with DAPI, and mounted on slides.

Fluorescence images were acquired using an EM-CCD camera (Andor) mounted on an inverted microscopy (IX81, Olympus) or using a confocal laser scanning microscopy (Zeiss LSM510). For quantification, regions of interest (ROIs) were set on neuronal nuclei, which were defined by DAPI and MAP2 staining, for

phosphoCREB-immunoreactivity (IR) (n = 89 - 154 neurons per condition), and on dendritic spines defined based on total CaMKIIα-IR for phosphoCaMKII (n = 127-203 spines from 9-12 neurons). Average intensity in each ROI was calculated using the MetaMorph software (Universal Imaging) and dose-dependent curves were drawn using the Prism software (GraphPad). The values under TTX condition and stimulation without drugs (DMSO only) were defined as basal (0 %) and maximum activities (100 %), respectively. The image analyses were done in a blind manner.

Figure S1 Dose-related effects of KN-93 on phosphorylation of CaMKII and CREB.

A, Effects of kinase inhibitors on phospho-CaMKII in dissociated hippocampal culture. Neurons were stimulated with bath application of glutamate/glycine in the presence of KN- 93. Phospho-CaMKII immunoreactivity in dendritic spines was measured for quantification. Framed areas in upper panels were expanded and pCaMKII channel was shown at the bottom in a pseudocolour scale.

B, Effects of kinase inhibitors on phospho-CREB in dissociated hippocampal culture. Neurons were stimulated with bath application of glutamate/glycine in the presence of KN-93. Phospho-CREB immunoreactivity was quantified in neuronal nuclei that were identified with MAP2 and DAPI staining. The pCREB channel was separately shown in a pseudocolour scale below.

C and D, Differential dose-responses of KN-93 on distinct CaMK pathways in culture neurons. C, Effects of KN-93 on CaMKII autophosphorylation at Thr286. Dissociated hippocampal cultures were treated with a series of concentrations of KN-93, stimulated with glutamate, and immunostained. Immunoreactivity for pCaMKII was quantified in dendritic spines and displayed as a function of KN-93 concentration. The ordinate represents basal (no stimulation, 0 %) to maximum (stimulated without inhibitors, 100%) activities. D, Effects of KN-93 on CREB phosphorylation at Ser133. Suppression of pCREB immunoreactivity in the neuronal nuclei was displayed. Note greater sensitivity of KN-93 for CaMKII.

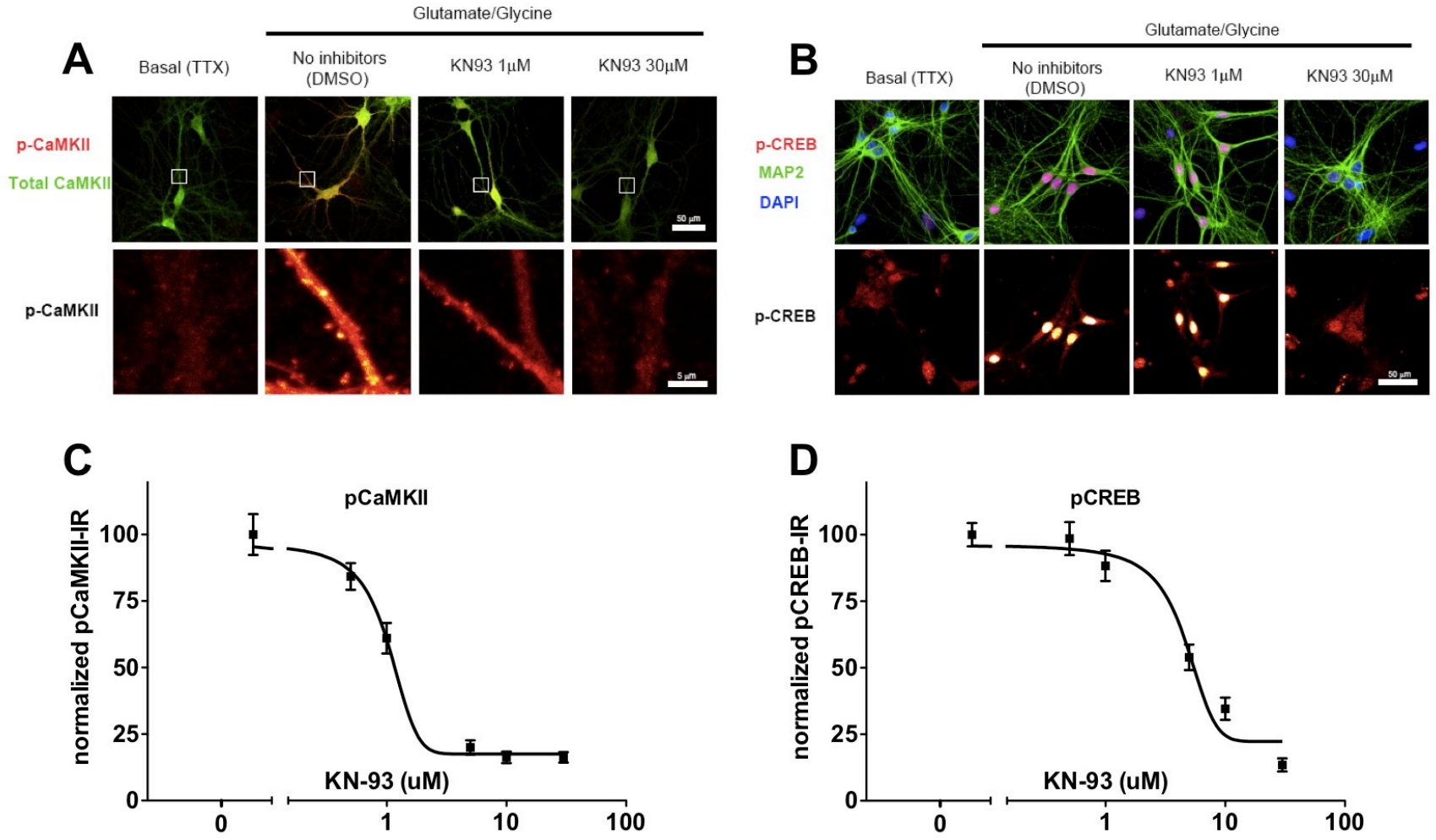
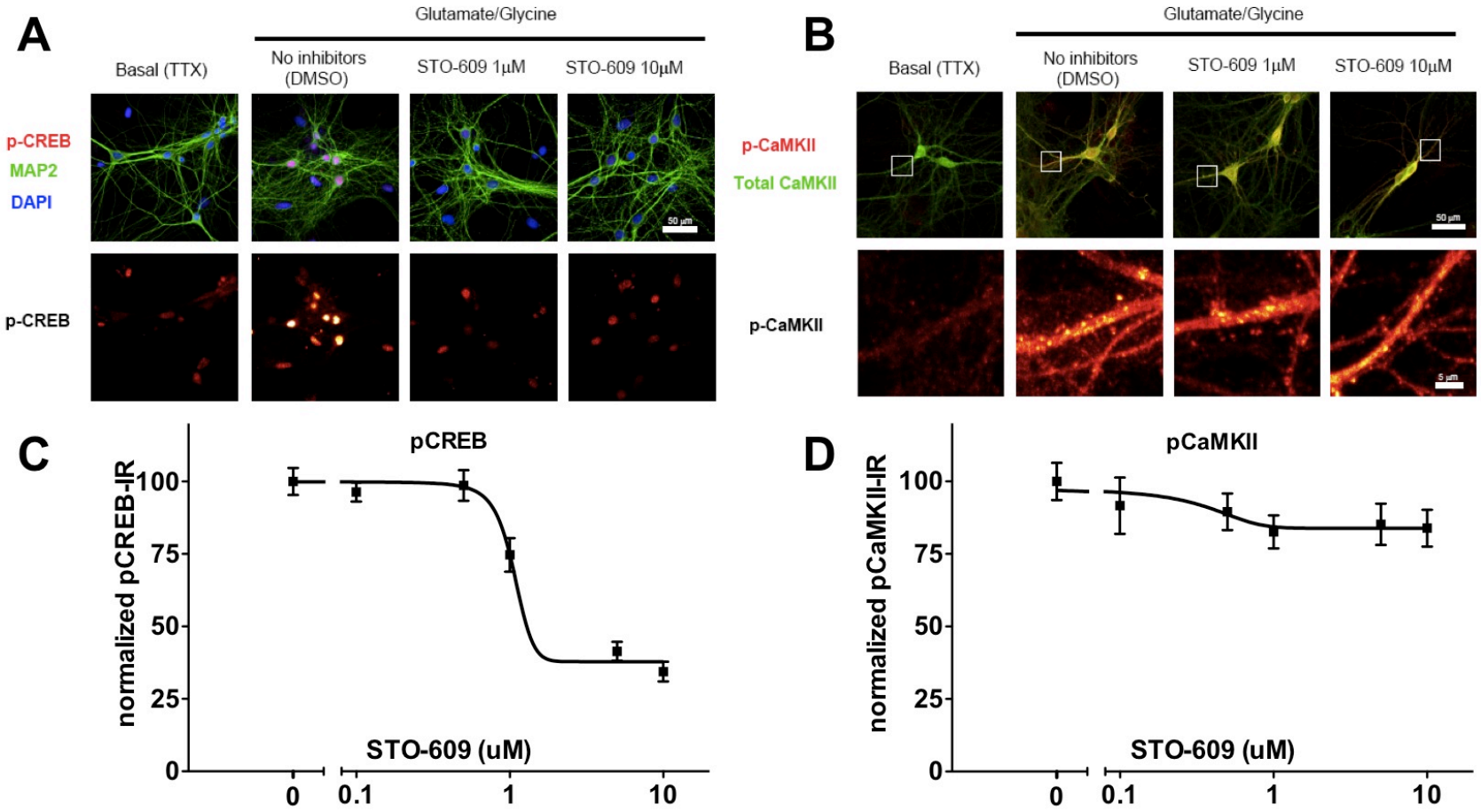

A
Glutamate/Glycine
Basal (TTX)
No inhibitors (DMSO)
KN93 1µM
KN93 30µM
p-CaMKII
Total CaMKII
p-CaMKII
50 µm
5 µm
B
Glutamate/Glycine
Basal (TTX)
No inhibitors (DMSO)
KN93 1µM
KN93 30µM
p-CREB
MAP2
DAPI
p-CREB
50 µm
C
pCaMKII
normalized pCaMKII-IR
100
75
50
25
0
KN-93 (uM)
0
1
10
100
D
pCREB
normalized pCREB-IR
100
75
50
25
0
KN-93 (uM)
0
1
10
100

Figure S2. Dose-related effects of STO-609 on phosphorylation of CaMKII and CREB.

A. *Effects of STO-609 on phospho-CREB in dissociated hippocampal culture*. Neurons were stimulated with bath application of glutamate/glycine in the presence of STO-609. Phospho-CREB immunoreactivity was quantified in neuronal nuclei that were identified with MAP2 and DAPI staining. The pCREB channel was separately shown in a pseudocolour scale below.

B. *Effects of STO-609 on phospho-CaMKII in dissociated hippocampal culture*. Neurons were stimulated with bath application of glutamate/glycine in the presence of STO-609. Phospho- CaMKII immunoreactivity in dendritic spines was measured for quantification. Framed areas in upper panels were expanded and pCaMKII channel was shown at the bottom in a pseudocolour scale.

C and D, Differential dose-responses of STO-609 on distinct CaMK pathways in culture neurons. C, Effects of STO-609 on CaMKII autophosphorylation at Thr286. D, Effects of STO-609 on CREB phosphorylation at Ser133.


A
Glutamate/Glycine
Basal (TTX)
No inhibitors (DMSO)
STO-609 1μM
STO-609 10μM
p-CREB
MAP2
DAPI
p-CREB
50 μm
B
Glutamate/Glycine
Basal (TTX)
No inhibitors (DMSO)
STO-609 1μM
STO-609 10μM
p-CaMKII
Total CaMKII
p-CaMKII
50 μm
5 μm
C
pCREB
normalized pCREB-IR
100
75
50
25
0
STO-609 (uM)
0
0.1
1
10
D
pCaMKII
normalized pCaMKII-IR
100
75
50
25
0
STO-609 (uM)
0
0.1
1
10

Chapter 9

Figure S3. The event arena room and training data (chapter 9).

One trial place learning in the event arena. (a) The photo, taken with wide-angle lens, presents the setting of the event arena room. (b) The errors that the animals made at the choice phase gradually declined after 17 training days. The horizontal dash line represents the chance level. (Linear trend, $F(1,15)=11.5$, $p < 0.01$). Data are presented in mean +/–s. e. m.

a

b

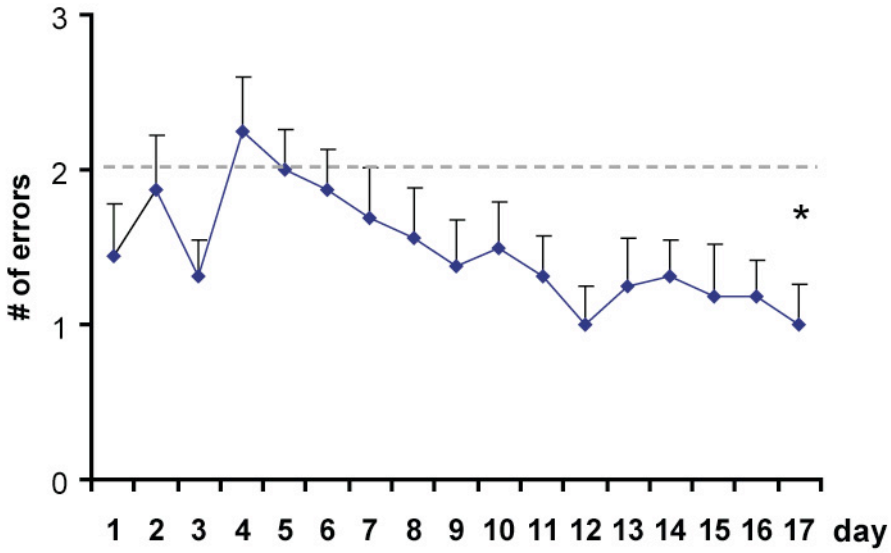


Figure S4. Place learning strategy in the event arena.

A, Animals received 1 pellet at encoding phase. Thirty minutes later, the probe trial started from the same start box as encoding, from a different start box, or from the same start box with the intra-maze landmarks removed.

B, Animals performed significantly better than chance in any of the conditions, suggesting the animals did not mainly rely on egocentric strategy or intra-maze cues. * indicates significant difference from chance, 20% ($p<0.05$). Data are presented in mean +/– s. e. m. (n = 11).

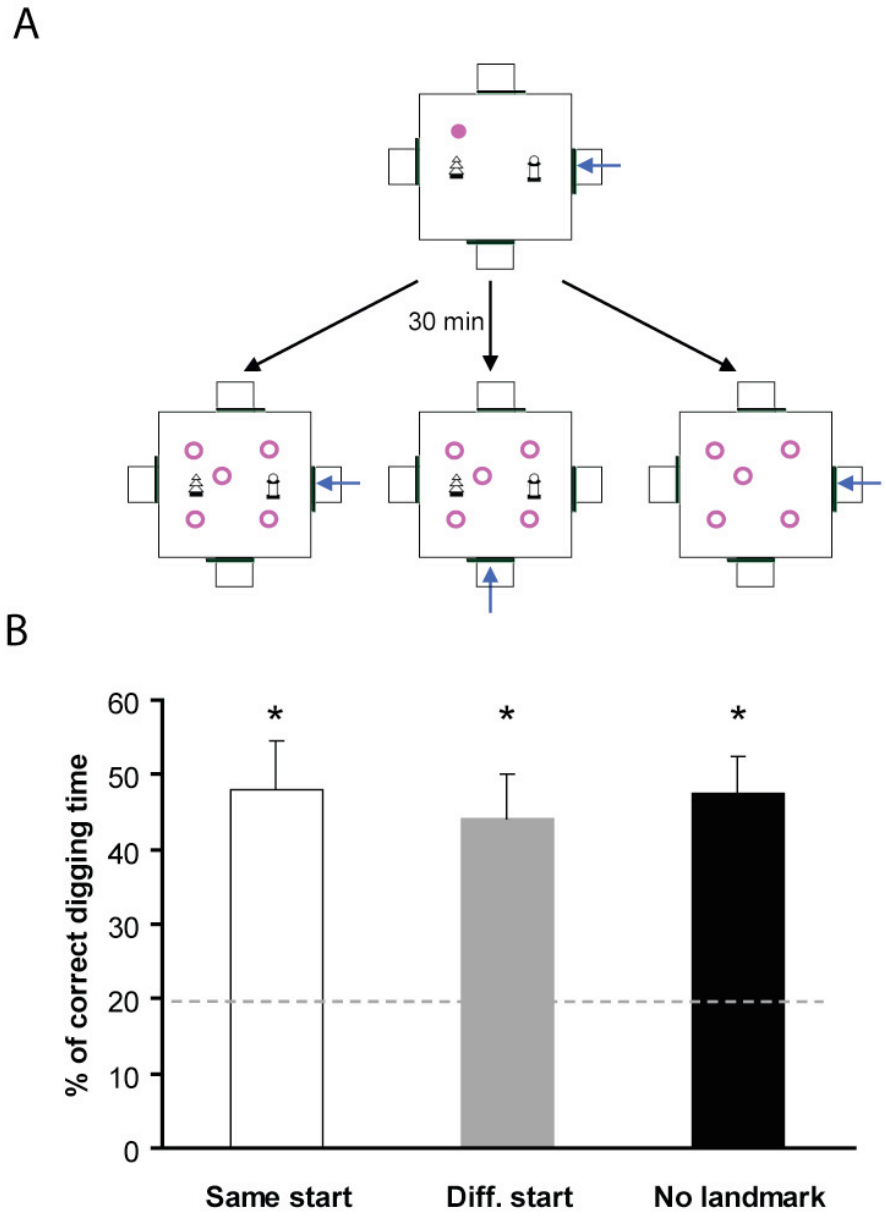
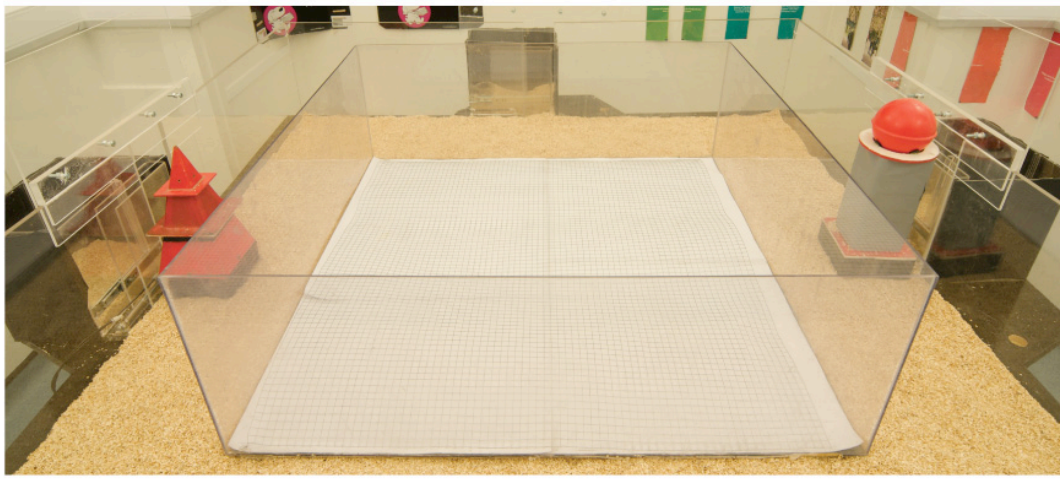
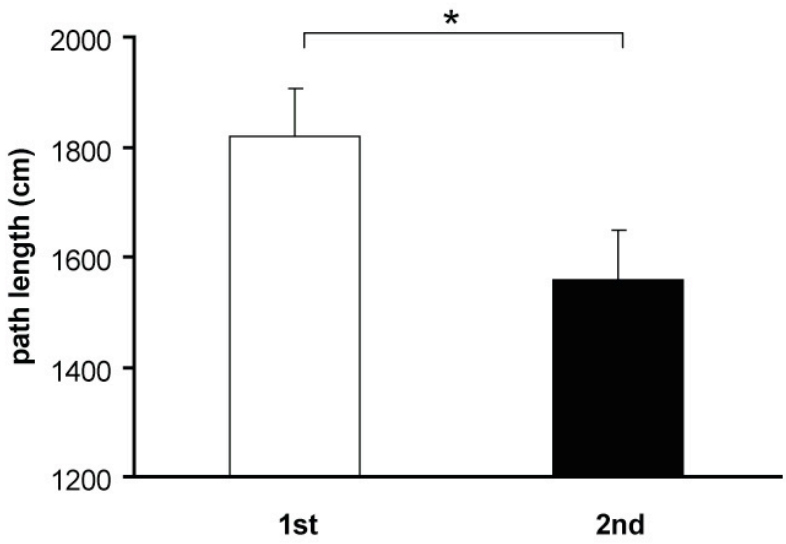


Figure S6. The exploration of a novel box.

A



B

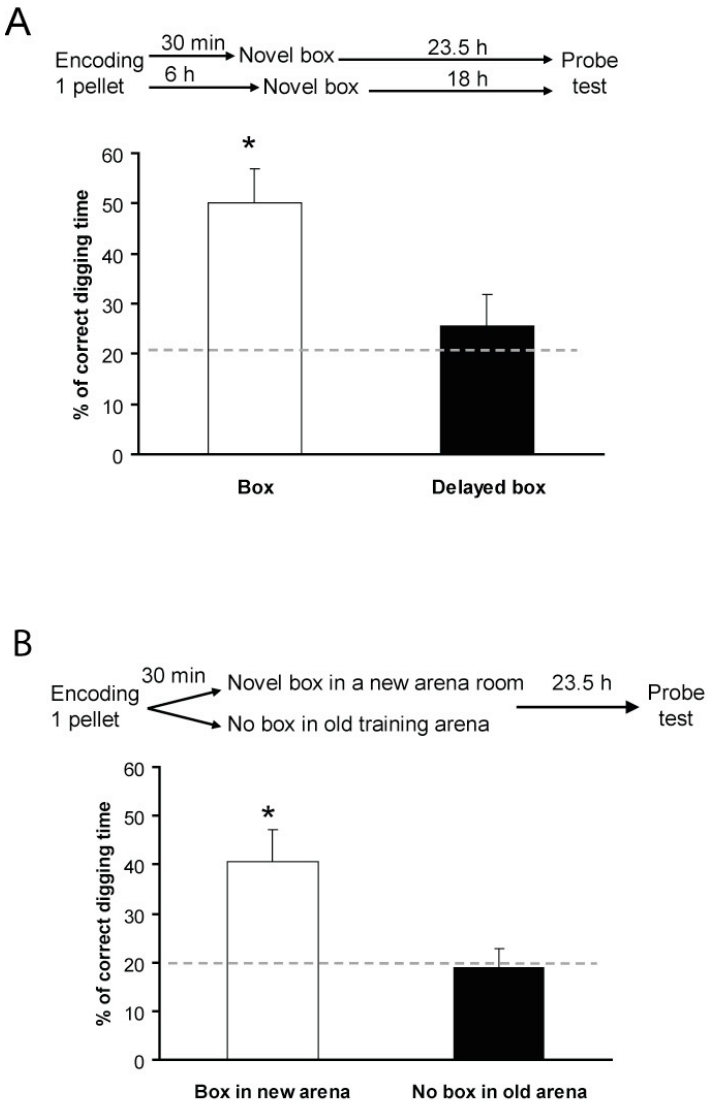


Exploration in a novel box. **(a)** This photo shows an example of the boxes that were used for exploration. In this case, the floor was composed of metal mesh wires on top of white cupboard paper and the walls were transparent. **(b)** Animals travelled in the box more during the first exposure than during the second exposure. * indicates significant difference ($p<0.05$). Data are presented in mean +/– s.e.m.

Figure S7. Additional tests on the novel box exploration.

A, The introduction of a 6 h delay between encoding and exploration in a novel box made the performance drop to chance level. * indicates significant difference from chance, 20% ($p<0.05$) (n = 11).

B, Novel box exploration is critical for the memory persistence. After encoding, exploration in a novel box in a new room, different from the old training room, was sufficient to facilitate the memory persistence, as shown by a significantly better than chance performance. Exploration in the training arena without a novel box was insufficient to maintain the 1-pellet memory on the next day, as shown by chance-level performance. * indicates significant difference from chance. Data are presented in mean +/– s.e.m. (n = 11).



Appendix 2: List of Figures

Bibliography

Abel T, Nguyen PV, Barad M, Deuel TA, Kandel ER, Bourtchouladze R. Genetic demonstration of a role for PKA in the late phase of LTP and in hippocampus-based long-term memory. Cell, 1997; 88: 615-26.

Abraham WC. Metaplasticity: tuning synapses and networks for plasticity. Nat Rev Neurosci, 2008; 9: 387.

Abraham WC, Logan B, Greenwood JM, Dragunow M. Induction and experience-dependent consolidation of stable long-term potentiation lasting months in the hippocampus. J Neurosci, 2002; 22: 9626-34.

Abraham WC, Williams JM. LTP maintenance and its protein synthesis-dependence. Neurobiol Learn Mem, 2008; 89: 260-8.

Abraham WC, Williams JM. Properties and mechanisms of LTP maintenance. Neuroscientist, 2003; 9: 463-74.

Ackermann M, Matus A. Activity-induced targeting of profilin and stabilization of dendritic spine morphology. Nat Neurosci, 2003; 6: 1194-200.

Acquas E, Wilson C, Fibiger HC. Conditioned and unconditioned stimuli increase frontal cortical and hippocampal acetylcholine release: effects of novelty, habituation, and fear. J Neurosci, 1996; 16: 3089-96.

Aiba A, Kano M, Chen C, Stanton ME, Fox GD, Herrup K, Zwingman TA, Tonegawa S. Deficient cerebellar long-term depression and impaired motor learning in mGluR1 mutant mice. Cell, 1994; 79: 377-88.

Aitken PG, Breese GR, Dudek FF, Edwards F, Espanol MT, Larkman PM, Lipton P, Newman GC, Nowak TS, Panizzon KL, Raley-Susman KM, Reid KH, Rice ME, Sarvey JM, Schoepp DD, Segal M, Taylor CP, Teyler TJ, Voulalas PJ. Preparative methods for brain slices: a discussion. Journal of Neuroscience Methods, 1995; 59: 139-49.

Akirav I, Kozenicky M, Tal D, Sandi C, Venero C, Richter-Levin G. A Facilitative Role for Corticosterone in the Acquisition of a Spatial Task Under Moderate Stress. Learn. Mem., 2004; 11: 188-95.

Alarcon JM, Barco A, Kandel ER. Capture of the Late Phase of Long-Term Potentiation within and across the Apical and Basilar Dendritic Compartments of CA1 Pyramidal Neurons: Synaptic Tagging Is Compartment Restricted. J. Neurosci., 2006; 26: 256-64.

Amaral DG. The Rat Nervous System, 3rd ed. Academic Press, 2004.

Andrasfalvy BK, Magee JC. Changes in AMPA receptor currents following LTP induction on rat CA1 pyramidal neurones. J Physiol, 2004; 559: 543-54.

Antonova I, Arancio O, Trillat AC, Wang HG, Zablow L, Udo H, Kandel ER, Hawkins RD. Rapid increase in clusters of presynaptic proteins at onset of long-lasting potentiation. Science, 2001; 294: 1547-50.

Applegate MD, Kerr DS, Landfield PW. Redistribution of synaptic vesicles during long-term potentiation in the hippocampus. Brain Res, 1987; 401: 401-6.

Arai A, Lynch G. Antagonists of the Platelet-activating Factor Receptor Block Long-term Potentiation in Hippocampal Slices. Eur J Neurosci, 1992; 4: 411-9.

Arami S, Jucker M, Schachner M, Welzl H. The effect of continuous intraventricular infusion of L1 and NCAM antibodies on spatial learning in rats. Behav Brain Res, 1996; 81: 81-7.

Atkins CM, Davare MA, Oh MC, Derkach V, Soderling TR. Bidirectional regulation of cytoplasmic polyadenylation element-binding protein phosphorylation by Ca2+/calmodulin-dependent protein kinase II and protein phosphatase 1 during hippocampal long-term potentiation. J Neurosci, 2005; 25: 5604-10.

Atwood HL, Karunanithi S. Diversification of synaptic strength: presynaptic elements. Nat Rev Neurosci, 2002; 3: 497-516.

Avital A, Segal M, Richter-Levin G. Contrasting Roles of Corticosteroid Receptors in Hippocampal Plasticity. J. Neurosci., 2006; 26: 9130-4.

Bach ME, Barad M, Son H, Zhuo M, Lu YF, Shih R, Mansuy I, Hawkins RD, Kandel ER. Age-related defects in spatial memory are correlated with defects in the late phase of hippocampal long-term potentiation in vitro and are attenuated by drugs that enhance the cAMP signaling pathway. Proc Natl Acad Sci U S A, 1999; 96: 5280-5.

Bacskai BJ, Hochner B, Mahaut-Smith M, Adams SR, Kaang BK, Kandel ER, Tsien RY. Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. Science, 1993; 260: 222-6.

Bagal AA, Kao JP, Tang CM, Thompson SM. Long-term potentiation of exogenous glutamate responses at single dendritic spines. Proc Natl Acad Sci U S A, 2005; 102: 14434-9.

Bailey CH, Chen M, Keller F, Kandel ER. Serotonin-mediated endocytosis of apCAM: an early step of learning-related synaptic growth in Aplysia. Science, 1992; 256: 645-9.

Bailey CH, Kandel ER, Si K. The persistence of long-term memory; a molecular approach to self-sustaining changes in learning-induced synaptic growth. Neuron, 2004; 44: 49-57.

Ballarini F, Moncada D, Martinez MC, Alen N, Viola H. Behavioral tagging is a general mechanism of long-term memory formation. Proc Natl Acad Sci U S A, 2009; 106: 14599-604.

Balschun D, Wolfer DP, Gass P, Mantamadiotis T, Welzl H, Schutz G, Frey JU, Lipp HP. Does cAMP response element-binding protein have a pivotal role in hippocampal synaptic plasticity and hippocampus-dependent memory? J Neurosci, 2003; 23: 6304-14.

Bamburg JR. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu Rev Cell Dev Biol, 1999; 15: 185-230.

Bannerman DM, Good MA, Butcher SP, Ramsay M, Morris RG. Distinct components of spatial learning revealed by prior training and NMDA receptor blockade. Nature, 1995; 378: 182-6.

Barad M, Bourtchouladze R, Winder DG, Golan H, Kandel E. Rolipram, a type IV-specific phosphodiesterase inhibitor, facilitates the establishment of long-lasting long-term potentiation and improves memory. PNAS, 1998; 95: 15020-5.

Baranes D, Lederfein D, Huang YY, Chen M, Bailey CH, Kandel ER. Tissue plasminogen activator contributes to the late phase of LTP and to synaptic growth in the hippocampal mossy fiber pathway. Neuron, 1998; 21: 813-25.

Barco A, Alarcon JM, Kandel ER. Expression of constitutively active CREB protein facilitates the late phase of long-term potentiation by enhancing synaptic capture. Cell, 2002; 108: 689-703.

Bashir ZI, Bortolotto ZA, Davies CH, Berretta N, Irving AJ, Seal AJ, Henley JM, Jane DE, Watkins JC, Collingridge GL. Induction of LTP in the hippocampus needs synaptic activation of glutamate metabotropic receptors. Nature, 1993; 363: 347-50.

Bashir ZI, Collingridge GL. An investigation of depotentiation of long-term potentiation in the CA1 region of the hippocampus. Experimental Brain Research, 1994; V79: 437-43.

Bast T, da Silva BM, Morris RG. Distinct contributions of hippocampal NMDA and AMPA receptors to encoding and retrieval of one-trial place memory. J Neurosci, 2005; 25: 5845-56.

Bastrikova N, Gardner GA, Reece JM, Jeromin A, Dudek SM. Synapse elimination accompanies functional plasticity in hippocampal neurons. Proc Natl Acad Sci U S A, 2008; 105: 3123-7.

Bayazitov IT, Richardson RJ, Fricke RG, Zakharenko SS. Slow presynaptic and fast postsynaptic components of compound long-term potentiation. J Neurosci, 2007; 27: 11510-21.

Bayer KU, De Koninck P, Leonard AS, Hell JW, Schulman H. Interaction with the NMDA receptor locks CaMKII in an active conformation. Nature, 2001; 411: 801-5.

Bayer KU, LeBel E, McDonald GL, O'Leary H, Schulman H, De Koninck P. Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. J Neurosci, 2006; 26: 1164-74.

Bear MF, Abraham WC. Long-term depression in hippocampus. Annu Rev Neurosci, 1996; 19: 437-62.

Becker CG, Artola A, Gerardy-Schahn R, Becker T, Welzl H, Schachner M. The polysialic acid modification of the neural cell adhesion molecule is involved in spatial learning and hippocampal long-term potentiation. J Neurosci Res, 1996; 45: 143-52.

Bekinschtein P, Cammarota M, Igaz LM, Bevilaqua LR, Izquierdo I, Medina JH. Persistence of long-term memory storage requires a late protein synthesis- and BDNF- dependent phase in the hippocampus. Neuron, 2007; 53: 261-77.

Benson DL, Schnapp LM, Shapiro L, Huntley GW. Making memories stick: cell-adhesion molecules in synaptic plasticity. Trends Cell Biol, 2000; 10: 473-82.

Bito H, Deisseroth K, Tsien RW. CREB phosphorylation and dephosphorylation: A Ca2+- and stimulus duration-dependent switch for hippocampal gene expression. Cell, 1996; 87: 1203-14.

Bliss T, Lömo T. Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. J. Physiol., 1973; 232: 331-56.

Bliss TV, Collingridge GL. A synaptic model of memory: long-term potentiation in the hippocampus. Nature, 1993; 361: 31-9.

Blitzer RD, Wong T, Nouranifar R, Iyengar R, Landau EM. Postsynaptic cAMP pathway gates early LTP in hippocampal CA1 region. Neuron, 1995; 15: 1403-14.

Bloomer WA, VanDongen HM, VanDongen AM. Arc/Arg3.1 translation is controlled by convergent N-methyl-D-aspartate and Gs-coupled receptor signaling pathways. J Biol Chem, 2008; 283: 582-92.

Bolshakov VY, Golan H, Kandel ER, Siegelbaum SA. Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3-CA1 synapses in the hippocampus. Neuron, 1997; 19: 635-51.

Bortolotto ZA, Bashir ZI, Davies CH, Collingridge GL. A molecular switch activated by metabotropic glutamate receptors regulates induction of long-term potentiation. Nature, 1994; 368: 740-3.

Bortolotto ZA, Collingridge GL. Involvement of calcium/calmodulin-dependent protein kinases in the setting of a molecular switch involved in hippocampal LTP. Neuropharmacology, 1998; 37: 535-44.

Bozdagi O, Shan W, Tanaka H, Benson DL, Huntley GW. Increasing Numbers of Synaptic Puncta during Late-Phase LTP: N-Cadherin Is Synthesized, Recruited to Synaptic Sites, and Required for Potentiation. Neuron, 2000; 28: 245-59.

Brakeman PR, Lanahan AA, O'Brien R, Roche K, Barnes CA, Huganir RL, Worley PF. Homer: a protein that selectively binds metabotropic glutamate receptors. Nature, 1997; 386: 284-8.

Bramham CR. Local protein synthesis, actin dynamics, and LTP consolidation. Curr Opin Neurobiol, 2008; 18: 524-31.

Bramham CR, Southard T, Ahlers ST, Sarvey JM. Acute cold stress leading to elevated corticosterone neither enhances synaptic efficacy nor impairs LTP in the dentate gyrus of freely moving rats. Brain Research, 1998; 789: 245-55.

Braun AP, Schulman H. The multifunctional calcium/calmodulin-dependent protein kinase: from form to function. Annual review of physiology, 1995; 57: 417-45.

Brown JT, Randall AD. Activity-dependent depression of the spike after-depolarization generates long-lasting intrinsic plasticity in hippocampal CA3 pyramidal neurons. J Physiol, 2009; 587: 1265-81.

Brown R, Kulik J. Flashbulb memories. Cognition, 1977; 5: 73-99.

Buchanan KA, Mellor JR. The development of synaptic plasticity induction rules and the requirement for postsynaptic spikes in rat hippocampal CA1 pyramidal neurones. J Physiol, 2007; 585: 429-45.

Buchs PA, Muller D. Induction of long-term potentiation is associated with major ultrastructural changes of activated synapses. Proc Natl Acad Sci U S A, 1996; 93: 8040-5.

Cahill L, Prins B, Weber M, McGaugh JL. Beta-adrenergic activation and memory for emotional events. Nature, 1994; 371: 702-4.

Carlisle HJ, Fink AE, Grant SG, O'Dell TJ. Opposing effects of PSD-93 and PSD-95 on long-term potentiation and spike timing-dependent plasticity. J Physiol, 2008a; 586: 5885-900.

Carlisle HJ, Kennedy MB. Spine architecture and synaptic plasticity. Trends in Neurosciences, 2005; 28: 182-7.

Carlisle HJ, Manzerra P, Marcora E, Kennedy MB. SynGAP regulates steady-state and activity-dependent phosphorylation of cofilin. J Neurosci, 2008b; 28: 13673-83.

Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, Bailey CH, Kandel ER. A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell, 1999; 99: 221-37.

Castellucci VF, Kandel ER, Schwartz JH, Wilson FD, Nairn AC, Greengard P. Intracellular injection of t he catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioral sensitization in Aplysia. Proc Natl Acad Sci U S A, 1980; 77: 7492-6.

Catterall WA, Few AP. Calcium channel regulation and presynaptic plasticity. Neuron, 2008; 59: 882-901.

Cavallaro S, Meiri N, Yi CL, Musco S, Ma W, Goldberg J, Alkon DL. Late memory-related genes in the hippocampus revealed by RNA fingerprinting. Proc Natl Acad Sci U S A, 1997; 94: 9669-73.

Chan C-S, Levenson JM, Mukhopadhyay PS, Zong L, Bradley A, Sweatt JD, Davis RL. {alpha}3-Integrins are required for hippocampal long-term potentiation and working memory. Learn. Mem., 2007; 14: 606-15.

Chan CS, Weeber EJ, Kurup S, Sweatt JD, Davis RL. Integrin requirement for hippocampal synaptic plasticity and spatial memory. J Neurosci, 2003; 23: 7107-16.

Chang FL, Greenough WT. Transient and enduring morphological correlates of synaptic activity and efficacy change in the rat hippocampal slice. Brain Res, 1984; 309: 35-46.

Chaouloff F, Hemar A, Manzoni O. Acute Stress Facilitates Hippocampal CA1 Metabotropic Glutamate Receptor-Dependent Long-Term Depression. J. Neurosci., 2007; 27: 7130-5.

Chawla S, Hardingham GE, Quinn DR, Bading H. CBP: a signal-regulated transcriptional coactivator controlled by nuclear calcium and CaM kinase IV. Science, 1998; 281: 1505-9.

Chen H-X, Otmakhov N, Strack S, Colbran RJ, Lisman JE. Is persistent activity of calcium/calmodulin-dependent kinase required for the maintenance of LTP? J Neurophysiol, 2001; 85: 1368-76.

Chen HJ, Rojas-Soto M, Oguni A, Kennedy MB. A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II. Neuron, 1998; 20: 895-904.

Chen Z, Fujii S, Ito K, Kato H, Kaneko K, Miyakawa H. Activation of dopamine D1 receptors enhances long-term depression of synaptic transmission induced by low frequency stimulation in rat hippocampal CA1 neurons. Neurosci Lett, 1995; 188: 195-8.

Chetkovich DM, Sweatt JD. NMDA receptor activation increases cyclic AMP in area CA1 of the hippocampus via calcium/calmodulin stimulation of adenylyl cyclase. J Neurochem, 1993; 61: 1933-42.

Chowdhury S, Shepherd JD, Okuno H, Lyford G, Petralia RS, Plath N, Kuhl D, Huganir RL, Worley PF. Arc/Arg3.1 Interacts with the Endocytic Machinery to Regulate AMPA Receptor Trafficking. Neuron, 2006; 52: 445-59.

Christofi G, Nowicky AV, Bolsover SR, Bindman LJ. The postsynaptic induction of nonassociative long-term depression of excitatory synaptic transmission in rat hippocampal slices. J Neurophysiol, 1993; 69: 219-29.

Christopherson KS, Hillier BJ, Lim WA, Bredt DS. PSD-95 assembles a ternary complex with the N-methyl-D-aspartic acid receptor and a bivalent neuronal NO synthase PDZ domain. J Biol Chem, 1999; 274: 27467-73.

Cingolani LA, Goda Y. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nat Rev Neurosci, 2008; 9: 344-56.

Clayton DF. The genomic action potential. Neurobiol Learn Mem, 2000; 74: 185-216.

Clements MP, Bliss TVP, Lynch MA. Increase in arachidonic acid concentration in a postsynaptic membrane fraction following the induction of long-term potentiation in the dentate gyrus. Neuroscience, 1991; 45: 379-89.

Coba MP, Valor LM, Kopanitsa MV, Afinowi NO, Grant SG. Kinase networks integrate profiles of NMDA receptor mediated gene expression in hippocampus. J Biol Chem, 2008.

Cobb MH, Goldsmith EJ. How MAP kinases are regulated. J Biol Chem, 1995; 270: 14843-6.

Collingridge G, Kehl S, McLennan H. Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. J. Physiol., 1983; 334: 33-46.

Croning MDR, Haddad GG. Comparison of brain slice chamber designs for investigations of oxygen deprivation in vitro. Journal of Neuroscience Methods, 1998; 81: 103-11.

da Silva B, Bast T. Dissecting episodic-like memory in rats: One-trial place memory in an event arena. 16th BNA meeting, Brighton, UK, 2004.

Dalva MB, McClelland AC, Kayser MS. Cell adhesion molecules: signalling functions at the synapse. 2007; advanced online publication.

Davies SN, Collingridge GL. Role of excitatory amino acid receptors in synaptic transmission in area CA1 of rat hippocampus. Proc R Soc Lond B Biol Sci, 1989; 236: 373-84.

Davies SN, Lester RA, Reymann KG, Collingridge GL. Temporally distinct pre- and post-synaptic mechanisms maintain long-term potentiation. Nature, 1989; 338: 500-3.

Davis CD, Jones FL, Derrick BE. Novel environments enhance the induction and maintenance of long-term potentiation in the dentate gyrus. J Neurosci, 2004; 24: 6497-506.

Davis HP, Squire LR. Protein synthesis and memory: a review. Psychol Bull, 1984; 96: 518-59.

Davis S, Vanhoutte P, Pages C, Caboche J, Laroche S. The MAPK/ERK Cascade Targets Both Elk-1 and cAMP Response Element-Binding Protein to Control Long-Term Potentiation-Dependent Gene Expression in the Dentate Gyrus In Vivo. J. Neurosci., 2000; 20: 4563-72.

de Kloet ER. Hormones, brain and stress. Endocr Regul, 2003; 37: 51-68.

Deisseroth K, Bito H, Tsien RW. Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron, 1996; 16: 89-101.

Deisseroth K, Mermelstein PG, Xia H, Tsien RW. Signaling from synapse to nucleus: the logic behind the mechanisms. Curr Opin Neurobiol, 2003; 13: 354-65.

DeMaria CD, Soong TW, Alseikhan BA, Alvania RS, Yue DT. Calmodulin bifurcates the local Ca2+ signal that modulates P/Q-type Ca2+ channels. Nature, 2001; 411: 484-9.

Derkach V, Barria A, Soderling TR. Ca2+/calmodulin-kinase II enhances channel conductance of alpha -amino-3-hydroxy-5-methyl-4-isoxazolepropionate type glutamate receptors. PNAS, 1999; 96: 3269-74.

Desmond NL, Levy WB. Changes in the numerical density of synaptic contacts with long-term potentiation in the hippocampal dentate gyrus. J Comp Neurol, 1986a; 253: 466-75.

Desmond NL, Levy WB. Changes in the postsynaptic density with long-term potentiation in the dentate gyrus. J Comp Neurol, 1986b; 253: 476-82.

Desmond NL, Levy WB. Synaptic correlates of associative potentiation/depression: an ultrastructural study in the hippocampus. Brain Res, 1983; 265: 21-30.

Deupree DL, Turner DA, Watters CL. Spatial performance correlates with in vitro potentiation in young and aged Fischer 344 rats. Brain Res, 1991; 554: 1-9.

Diamond D, Park C, Woodson J. Stress generates emotional memories and retrograde amnesia by inducing an endogenous form of hippocampal LTP. Hippocampus, 2004; 14: 281-91.

Diamond DM, Bennett MC, Fleshner M, Rose GM. Inverted-U relationship between the level of peripheral corticosterone and the magnitude of hippocampal primed burst potentiation. Hippocampus, 1992; 2: 421-30.

Dityatev A, Schachner M. Extracellular matrix molecules and synaptic plasticity. Nat Rev Neurosci, 2003; 4: 456-68.

Dong C, Upadhya SC, Ding L, Smith TK, Hegde AN. Proteasome inhibition enhances the induction and impairs the maintenance of late-phase long-term potentiation. Learn Mem, 2008; 15: 335-47.

Doyle E, Nolan PM, Bell R, Regan CM. Hippocampal NCAM180 transiently increases sialylation during the acquisition and consolidation of a passive avoidance response in the adult rat. J Neurosci Res, 1992a; 31: 513-23.

Doyle E, Nolan PM, Bell R, Regan CM. Intraventricular infusions of anti-neural cell adhesion molecules in a discrete posttraining period impair consolidation of a passive avoidance response in the rat. J Neurochem, 1992b; 59: 1570-3.

Duffy SN, Nguyen PV. Postsynaptic Application of a Peptide Inhibitor of cAMP-Dependent Protein Kinase Blocks Expression of Long-Lasting Synaptic Potentiation in Hippocampal Neurons. J. Neurosci., 2003; 23: 1142-50.

Dunwiddie TV, Lynch G. The relationship between extracellular calcium concentrations and the induction of hippocampal long-term potentiation. Brain Research, 1979; 169: 103-10.

Earnest S, Khokhlatchev A, Albanesi JP, Barylko B. Phosphorylation of dynamin by ERK2 inhibits the dynamin-microtubule interaction. FEBS Lett, 1996; 396: 62-6.

Ehlers MD. Activity level controls postsynaptic composition and signaling via the ubiquitin-proteasome system. Nat Neurosci, 2003; 6: 231-42.

Ehlers MD. Molecular morphogens for dendritic spines. Trends in Neurosciences, 2002; 25: 64-7.

Eichenbaum H, Dudchenko P, Wood E, Shapiro M, Tanila H. The hippocampus, memory, and place cells: is it spatial memory or a memory space? Neuron, 1999; 23: 209-26.

Eichenbaum H, Stewart C, Morris RG. Hippocampal representation in place learning. J Neurosci, 1990; 10: 3531-42.

Elgersma Y, Sweatt JD, Giese KP. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. J Neurosci, 2004; 24: 8410-5.

Emptage N, Bliss TVP, Fine A. Single Synaptic Events Evoke NMDA Receptor-Mediated Release of Calcium from Internal Stores in Hippocampal Dendritic Spines. Neuron, 1999; 22: 115-24.

Engert F, Bonhoeffer T. Dendritic spine changes associated with hippocampal long-term synaptic plasticity. Nature, 1999; 399: 66-70.

Enoki R, Hu YL, Hamilton D, Fine A. Expression of long-term plasticity at individual synapses in hippocampus is graded, bidirectional, and mainly presynaptic: optical quantal analysis. Neuron, 2009; 62: 242-53.

Enslen H, Sun P, Brickey D, Soderling SH, Klamo E, Soderling TR. Characterization of Ca2+/calmodulin-dependent protein kinase IV. Role in transcriptional regulation. J Biol Chem, 1994; 269: 15520-7.

Enslen H, Tokumitsu H, Stork PJ, Davis RJ, Soderling TR. Regulation of mitogen-activated protein kinases by a calcium/calmodulin-dependent protein kinase cascade. Proc Natl Acad Sci U S A, 1996; 93: 10803-8.

Erondu NE, Kennedy MB. Regional distribution of type II Ca2+/calmodulin-dependent protein kinase in rat brain. J Neurosci, 1985; 5: 3270-7.

Errington ML, Bliss TV, Richter-Levin G, Yenk K, Doyere V, Laroche S. Stimulation at 1-5 Hz does not produce long-term depression or depotentiation in the hippocampus of the adult rat in vivo. J Neurophysiol, 1995; 74: 1793-9.

Esteban JA, Shi SH, Wilson C, Nuriya M, Huganir RL, Malinow R. PKA phosphorylation of AMPA receptor subunits controls synaptic trafficking underlying plasticity. Nat Neurosci, 2003; 6: 136-43.

Farooqui AA, Ong W-Y, Horrocks LA. Inhibitors of Brain Phospholipase A2 Activity: Their Neuropharmacological Effects and Therapeutic Importance for the Treatment of Neurologic Disorders. Pharmacol Rev, 2006; 58: 591-620.

Fiala JC, Kirov SA, Feinberg MD, Petrak LJ, George P, Goddard CA, Harris KM. Timing of neuronal and glial ultrastructure disruption during brain slice preparation and recovery in vitro. J Comp Neurol, 2003; 465: 90-103.

Fifkova E, Van Harreveld A. Long-lasting morphological changes in dendritic spines of dentate granular cells following stimulation of the entorhinal area. J Neurocytol, 1977; 6: 211-30.

Fischer M, Kaech S, Knutti D, Matus A. Rapid actin-based plasticity in dendritic spines. Neuron, 1998; 20: 847-54.

Foley AG, Hartz BP, Gallagher HC, Ronn LC, Berezin V, Bock E, Regan CM. A synthetic peptide ligand of neural cell adhesion molecule (NCAM) IgI domain prevents NCAM internalization and disrupts passive avoidance learning. J Neurochem, 2000; 74: 2607-13.

Fonseca R, Nagerl UV, Bonhoeffer T. Neuronal activity determines the protein synthesis dependence of long-term potentiation. Nature Neuroscience, 2006a; 9: 478-80.

Fonseca R, Nagerl UV, Morris RGM, Bonhoeffer T. Competing for memory: hippocampal LTP under regimes of reduced protein synthesis. Neuron, 2004; 44: 1011-20.

Fonseca R, Vabulas RM, Hartl FU, Bonhoeffer T, Nagerl UV. A Balance of Protein Synthesis and Proteasome-Dependent Degradation Determines the Maintenance of LTP. Neuron, 2006b; 52: 239-45.

Fox CJ, Russell KI, Wang YT, Christie BR. Contribution of NR2A and NR2B NMDA subunits to bidirectional synaptic plasticity in the hippocampus in vivo. Hippocampus, 2006.

Frey U, Huang YY, Kandel ER. Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. Science, 1993; 260: 1661-4.

Frey U, Krug M, Brodemann R, Reymann K, Matthies H. Long-term potentiation induced in dendrites separated from rat's CA1 pyramidal somata does not establish a late phase. Neuroscience Letters, 1989; 97: 135-9.

Frey U, Krug M, Reymann KG, Matthies H. Anisomycin, an inhibitor of protein synthesis, blocks late phases of LTP phenomena in the hippocampal CA1 region in vitro. Brain Res, 1988; 452: 57-65.

Frey U, Matthies H, Reymann KG. The effect of dopaminergic D1 receptor blockade during tetanization on the expression of long-term potentiation in the rat CA1 region in vitro. Neurosci Lett, 1991; 129: 111-4.

Frey U, Morris RGM. Synaptic tagging and long-term potentiation. Nature, 1997; 385: 533-6.

Frey U, Morris RGM. Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. Trends in Neurosciences, 1998a; 21: 181-8.

Frey U, Morris RGM. Weak before strong: dissociating synaptic tagging and plasticity-factor accounts of late-LTP. Neuropharmacology, 1998b; 37: 545-52.

Frey U, Muller M, Kuhl D. A different form of long-lasting potentiation revealed in tissue plasminogen activator mutant mice. J Neurosci, 1996; 16: 2057-63.

Frey U, Schroeder H, Matthies H. Dopaminergic antagonists prevent long-term maintenance of posttetanic LTP in the CA1 region of rat hippocampal slices. Brain Res, 1990; 522: 69-75.

Fukazawa Y, Saitoh Y, Ozawa F, Ohta Y, Mizuno K, Inokuchi K. Hippocampal LTP is accompanied by enhanced F-actin content within the dendritic spine that is essential for late LTP maintenance in vivo. Neuron, 2003; 38: 447-60.

Furuyashiki T, Arakawa Y, Takemoto-Kimura S, Bito H, Narumiya S. Multiple spatiotemporal modes of actin reorganization by NMDA receptors and voltage-gated Ca2+ channels. Proc Natl Acad Sci U S A, 2002; 99: 14458-63.

Gardiol A, Racca C, Triller A. Dendritic and postsynaptic protein synthetic machinery. J Neurosci, 1999; 19: 168-79.

Gasbarri A, Sulli A, Innocenzi R, Pacitti C, Brioni JD. Spatial memory impairment induced by lesion of the mesohippocampal dopaminergic system in the rat. Neuroscience, 1996; 74: 1037-44.

Geinisman Y. Perforated axospinous synapses with multiple, completely partitioned transmission zones: probable structural intermediates in synaptic plasticity. Hippocampus, 1993; 3: 417-33.

Geinisman Y, deToledo-Morrell L, Morrell F. Induction of long-term potentiation is associated with an increase in the number of axospinous synapses with segmented postsynaptic densities. Brain Res, 1991; 566: 77-88.

Gelinas JN, Nguyen PV. Beta-adrenergic receptor activation facilitates induction of a protein synthesis-dependent late phase of long-term potentiation. J Neurosci, 2005; 25: 3294-303.

Gelinas JN, Tenorio G, Lemon N, Abel T, Nguyen PV. Beta-adrenergic receptor activation during distinct patterns of stimulation critically modulates the PKA-dependence of LTP in the mouse hippocampus. Learn Mem, 2008; 15: 281-9.

Giese KP, Fedorov NB, Filipkowski RK, Silva AJ. Autophosphorylation at Thr286 of the alpha calcium-calmodulin kinase II in LTP and learning. Science, 1998; 279: 870-3.

Gong R, Tang SJ. Mitogen-activated protein kinase signaling is essential for activity-dependent dendritic protein synthesis. Neuroreport, 2006; 17: 1575-8.

Govindarajan A, Kelleher RJ, Tonegawa S. A clustered plasticity model of long-term memory engrams. 2006; 7: 575-83.

Granado N, Ortiz O, Suarez LM, Martin ED, Cena V, Solis JM, Moratalla R. D1 but not D5 dopamine receptors are critical for LTP, spatial learning, and LTP-Induced arc and zif268 expression in the hippocampus. Cereb Cortex, 2008; 18: 1-12.

Guire ES, Oh MC, Soderling TR, Derkach VA. Recruitment of calcium-permeable AMPA receptors during synaptic potentiation is regulated by CaM-kinase I. J Neurosci, 2008; 28: 6000-9.

Gustafsson B, Wigstrom H, Abraham WC, Huang YY. Long-term potentiation in the hippocampus using depolarizing current pulses as the conditioning stimulus to single volley synaptic potentials. J Neurosci, 1987; 7: 774-80.

Guzowski JF, Lyford GL, Stevenson GD, Houston FP, McGaugh JL, Worley PF, Barnes CA. Inhibition of activity-dependent arc protein expression in the rat hippocampus impairs the maintenance of long-term potentiation and the consolidation of long-term memory. J Neurosci, 2000; 20: 3993-4001.

Haas HL, Schaerer B, Vosmansky M. A simple perfusion chamber for the study of nervous tissue slices in vitro. J Neurosci Methods, 1979; 1: 323-5.

Hallenbeck P, Vimr E, Yu F, Bassler B, Troy F. Purification and properties of a bacteriophage-induced endo-N- acetylneuraminidase specific for poly-alpha-2,8-sialosyl carbohydrate units. J. Biol. Chem., 1987; 262: 3553-61.

Halpain S. Actin and the agile spine: how and why do dendritic spines dance? Trends in Neurosciences, 2000; 23: 141-6.

Halpain S, Hipolito A, Saffer L. Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. J Neurosci, 1998; 18: 9835-44.

Han JH, Kushner SA, Yiu AP, Hsiang HL, Buch T, Waisman A, Bontempi B, Neve RL, Frankland PW, Josselyn SA. Selective erasure of a fear memory. Science, 2009; 323: 1492-6.

Hansel C, de Jeu M, Belmeguenai A, Houtman SH, Buitendijk GH, Andreev D, De Zeeuw CI, Elgersma Y. alphaCaMKII Is essential for cerebellar LTD and motor learning. Neuron, 2006; 51: 835-43.

Hardingham GE, Chawla S, Cruzalegui FH, Bading H. Control of recruitment and transcription-activating function of CBP determines gene regulation by NMDA receptors and L-type calcium channels. Neuron, 1999; 22: 789-98.

Hardingham GE, Cruzalegui FH, Chawla S, Bading H. Mechanisms controlling gene expression by nuclear calcium signals. Cell Calcium, 1998; 23: 131-4.

Harris KM. Structure, development, and plasticity of dendritic spines. Curr Opin Neurobiol, 1999; 9: 343-8.

Hayashi Y, Shi S-H, Esteban JeA, Piccini A, Poncer J-C, Malinow R. Driving AMPA Receptors into Synapses by LTP and CaMKII: Requirement for GluR1 and PDZ Domain Interaction. Science, 2000; 287: 2262-7.

Hebb DO. The organization of behavior : a neuropsychological theory. Wiley ;
Chapman & Hall: New York
London, 1949.

Hegde AN. Ubiquitin-proteasome-mediated local protein degradation and synaptic plasticity. Prog Neurobiol, 2004; 73: 311-57.

Hering H, Sheng M. Activity-dependent redistribution and essential role of cortactin in dendritic spine morphogenesis. J Neurosci, 2003; 23: 11759-69.

Hersi AI, Rowe W, Gaudreau P, Quirion R. Dopamine D1 receptor ligands modulate cognitive performance and hippocampal acetylcholine release in memory-impaired aged rats. Neuroscience, 1995; 69: 1067-74.

Hetman M, Kanning K, Cavanaugh JE, Xia Z. Neuroprotection by Brain-derived Neurotrophic Factor Is Mediated by Extracellular Signal-regulated Kinase and Phosphatidylinositol 3-Kinase. J. Biol. Chem., 1999; 274: 22569-80.

Heynen AJ, Quinlan EM, Bae DC, Bear MF. Bidirectional, activity-dependent regulation of glutamate receptors in the adult hippocampus in vivo. Neuron, 2000; 28: 527-36.

Hill M, Froc D, Fox C, Gorzalka B, Christie B. Prolonged cannabinoid treatment results in spatial working memory deficits and impaired long-term potentiation in the CA1 region of the hippocampus in vivo. Eur J Neurosci, 2004; 20: 859-63.

Ho N, Liauw JA, Blaeser F, Wei F, Hanissian S, Muglia LM, Wozniak DF, Nardi A, Arvin KL, Holtzman DM, Linden DJ, Zhuo M, Muglia LJ, Chatila TA. Impaired synaptic plasticity and cAMP response element-binding protein activation in Ca2+/calmodulin-dependent protein kinase type IV/Gr-deficient mice. J Neurosci, 2000; 20: 6459-72.

Ho OH, Delgado JY, O'Dell TJ. Phosphorylation of proteins involved in activity-dependent forms of synaptic plasticity is altered in hippocampal slices maintained in vitro. J Neurochem, 2004; 91: 1344-57.

Holscher C, McGlinchey L, Rowan MJ. L-AP4 (L-(+)-2-amino-4-phosphonobutyric acid) induced impairment of spatial learning in the rat is antagonized by MAP4 ((S)-2-amino-2-methyl-4-phosphonobutanoic acid). Behav Brain Res, 1996; 81: 69-79.

Honkura N, Matsuzaki M, Noguchi J, Ellis-Davies GC, Kasai H. The subspine organization of actin fibers regulates the structure and plasticity of dendritic spines. Neuron, 2008; 57: 719-29.

Horton AC, Racz B, Monson EE, Lin AL, Weinberg RJ, Ehlers MD. Polarized secretory trafficking directs cargo for asymmetric dendrite growth and morphogenesis. Neuron, 2005; 48: 757-71.

Hou Q, Gao X, Zhang X, Kong L, Wang X, Bian W, Tu Y, Jin M, Zhao G, Li B, Jing N, Yu L. SNAP-25 in hippocampal CA1 region is involved in memory consolidation. Eur J Neurosci, 2004; 20: 1593-603.

Huang F, Chotiner JK, Steward O. The mRNA for elongation factor 1alpha is localized in dendrites and translated in response to treatments that induce long-term depression. J Neurosci, 2005; 25: 7199-209.

Huang Y, Kandel E. D1/D5 Receptor Agonists Induce a Protein Synthesis-Dependent Late Potentiation in the CA1 Region of the Hippocampus. PNAS, 1995; 92: 2446-50.

Huang Y, Kandel E. Recruitment of long-lasting and protein kinase A-dependent long-term potentiation in the CA1 region of hippocampus requires repeated tetanization. Learn. Mem., 1994; 1: 74-.

Huang YS, Jung MY, Sarkissian M, Richter JD. N-methyl-D-aspartate receptor signaling results in Aurora kinase-catalyzed CPEB phosphorylation and alpha CaMKII mRNA polyadenylation at synapses. The EMBO journal, 2002; 21: 2139-48.

Huang YY, Bach ME, Lipp HP, Zhuo M, Wolfer DP, Hawkins RD, Schoonjans L, Kandel ER, Godfraind JM, Mulligan R, Collen D, Carmeliet P. Mice lacking the gene encoding tissue-type plasminogen activator show a selective interference with late-phase long-term potentiation in both Schaffer collateral and mossy fiber pathways. Proc Natl Acad Sci U S A, 1996; 93: 8699-704.

Huntley GW, Benson DL, Colman DR. Structural remodeling of the synapse in response to physiological activity. Cell, 2002; 108: 1-4.

Ihalainen JA, Riekkinen P, Jr., Feenstra MG. Comparison of dopamine and noradrenaline release in mouse prefrontal cortex, striatum and hippocampus using microdialysis. Neurosci Lett, 1999; 277: 71-4.

Impey S, Mark M, Villacres EC, Poser S, Chavkin C, Storm DR. Induction of CRE-mediated gene expression by stimuli that generate long-lasting LTP in area CA1 of the hippocampus. Neuron, 1996; 16: 973-82.

Isaac JT, Buchanan KA, Muller RU, Mellor JR. Hippocampal place cell firing patterns can induce long-term synaptic plasticity in vitro. J Neurosci, 2009; 29: 6840-50.

Ishida A, Kameshita I, Okuno S, Kitani T, Fujisawa H. A novel highly specific and potent inhibitor of calmodulin-dependent protein kinase II. Biochem Biophys Res Commun, 1995; 212: 806-12.

Ismailov I, Kalikulov D, Inoue T, Friedlander MJ. The kinetic profile of intracellular calcium predicts long-term potentiation and long-term depression. J Neurosci, 2004; 24: 9847-61.

Isomura Y, Fujiwara-Tsukamoto Y, Imanishi M, Nambu A, Takada M. Distance-dependent Ni(2+)-sensitivity of synaptic plasticity in apical dendrites of hippocampal CA1 pyramidal cells. J Neurophysiol, 2002; 87: 1169-74.

Jay TM. Dopamine: a potential substrate for synaptic plasticity and memory mechanisms. Progress in Neurobiology, 2003; 69: 375-90.

Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC. Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. Nat Neurosci, 2004; 7: 244-53.

Kandel ER. The molecular biology of memory storage: a dialog between genes and synapses. Biosci Rep, 2001; 21: 565-611.

Kandel ER, Spencer WA. Cellular neurophysiological approaches in the study of learning. Physiol Rev, 1968; 48: 65-134.

Kang H, Schuman EM. A Requirement for Local Protein Synthesis in Neurotrophin-Induced Hippocampal Synaptic Plasticity. Science, 1996; 273: 1402-6.

Kang H, Sun LD, Atkins CM, Soderling TR, Wilson MA, Tonegawa S. An important role of neural activity-dependent CaMKIV signaling in the consolidation of long-term memory. Cell, 2001; 106: 771-83.

Karpova A, Mikhaylova M, Thomas U, Knopfel T, Behnisch T. Involvement of protein synthesis and degradation in long-term potentiation of Schaffer collateral CA1 synapses. J Neurosci, 2006; 26: 4949-55.

Kato A, Ozawa F, Saitoh Y, Hirai K, Inokuchi K. vesl, a gene encoding VASP/Ena family related protein, is upregulated during seizure, long-term potentiation and synaptogenesis. FEBS Lett, 1997; 412: 183-9.

Kauderer BS, Kandel ER. Capture of a protein synthesis-dependent component of long-term depression. PNAS, 2000; 97: 13342-7.

Kawashima T, Okuno H, Nonaka M, Adachi-Morishima A, Kyo N, Okamura M, Takemoto-Kimura S, Worley PF, Bito H. Synaptic activity-responsive element in the Arc/Arg3.1 promoter essential for synapse-to-nucleus signaling in activated neurons. Proc Natl Acad Sci U S A, 2009; 106: 316-21.

Kelleher RJ, 3rd, Govindarajan A, Jung HY, Kang H, Tonegawa S. Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell, 2004a; 116: 467-79.

Kelleher RJ, 3rd, Govindarajan A, Tonegawa S. Translational regulatory mechanisms in persistent forms of synaptic plasticity. Neuron, 2004b; 44: 59-73.

Kelly A, Laroche S, Davis S. Activation of mitogen-activated protein kinase/extracellular signal-regulated kinase in hippocampal circuitry is required for

consolidation and reconsolidation of recognition memory. J Neurosci, 2003; 23: 5354-60.

Kemp A, Manahan-Vaughan D. Hippocampal long-term depression and long-term potentiation encode different aspects of novelty acquisition. PNAS, 2004.

Kemp N, Bashir ZI. Long-term depression: a cascade of induction and expression mechanisms. Prog Neurobiol, 2001; 65: 339-65.

Kennedy MB. Signal-processing machines at the postsynaptic density. Science, 2000; 290: 750-4.

Kennedy MJ, Ehlers MD. Organelles and trafficking machinery for postsynaptic plasticity. Annu Rev Neurosci, 2006; 29: 325-62.

Kim DY, Kim SH, Choi HB, Min C, Gwag BJ. High abundance of GluR1 mRNA and reduced Q/R editing of GluR2 mRNA in individual NADPH-diaphorase neurons. Mol Cell Neurosci, 2001; 17: 1025-33.

Kim JH, Liao D, Lau LF, Huganir RL. SynGAP: a synaptic RasGAP that associates with the PSD-95/SAP90 protein family. Neuron, 1998; 20: 683-91.

Kim JJ, Koo JW, Lee HJ, Han J-S. Amygdalar Inactivation Blocks Stress-Induced Impairments in Hippocampal Long-Term Potentiation and Spatial Memory. J. Neurosci., 2005; 25: 1532-9.

Kimura K, Sela S, Bouvier C, Grandy DK, Sidhu A. Differential coupling of D1 and D5 dopamine receptors to guanine nucleotide binding proteins in transfected GH4C1 rat somatomammotrophic cells. J Neurochem, 1995; 64: 2118-24.

Kitamura Y, Miyazaki A, Yamanaka Y, Nomura Y. Stimulatory effects of protein kinase C and calmodulin kinase II on N-methyl-D-aspartate receptor/channels in the postsynaptic density of rat brain. J Neurochem, 1993; 61: 100-9.

Kitchigina V, Vankov A, Harley C, Sara SJ. Novelty-elicited, noradrenaline-dependent enhancement of excitability in the dentate gyrus. Eur J Neurosci, 1997; 9: 41-7.

Klann E, Antion MD, Banko JL, Hou L. Synaptic Plasticity and Translation Initiation. Learn. Mem., 2004; 11: 365-72.

Kock N, Chatelain-Jardon R, Carmona J. An Experimental Study of Simulated Web-Based Threats and Their Impact on Knowledge Communication Effectiveness. Professional Communication, IEEE Transactions on, 2008; 51: 183-97.

Kohrmann M, Luo M, Kaether C, DesGroseillers L, Dotti CG, Kiebler MA. Microtubule-dependent recruitment of Staufen-green fluorescent protein into large

RNA-containing granules and subsequent dendritic transport in living hippocampal neurons. Mol Biol Cell, 1999; 10: 2945-53.

Komai S, Matsuyama T, Matsumoto K, Kato K, Kobayashi M, Imamura K, Yoshida S, Ugawa S, Shiosaka S. Neuropsin regulates an early phase of schaffer-collateral long-term potentiation in the murine hippocampus. Eur J Neurosci, 2000; 12: 1479-86.

Komiyama NH, Watabe AM, Carlisle HJ, Porter K, Charlesworth P, Monti J, Strathdee DJ, O'Carroll CM, Martin SJ, Morris RG, O'Dell TJ, Grant SG. SynGAP regulates ERK/MAPK signaling, synaptic plasticity, and learning in the complex with postsynaptic density 95 and NMDA receptor. J Neurosci, 2002; 22: 9721-32.

Kopec CD, Real E, Kessels HW, Malinow R. GluR1 links structural and functional plasticity at excitatory synapses. J Neurosci, 2007; 27: 13706-18.

Kovalchuk Y, Eilers J, Lisman J, Konnerth A. NMDA receptor-mediated subthreshold Ca(2+) signals in spines of hippocampal neurons. J Neurosci, 2000; 20: 1791-9.

Krapivinsky G, Medina I, Krapivinsky L, Gapon S, Clapham DE. SynGAP-MUPP1-CaMKII synaptic complexes regulate p38 MAP kinase activity and NMDA receptor-dependent synaptic AMPA receptor potentiation. Neuron, 2004; 43: 563-74.

Krucker T, Siggins GR, Halpain S. Dynamic actin filaments are required for stable long-term potentiation (LTP) in area CA1 of the hippocampus. Proceedings Of The National Academy Of Sciences Of The United States Of America, 2000; 97: 6856-61.

Krug M, Lossner B, Ott T. Anisomycin blocks the late phase of long-term potentiation in the dentate gyrus of freely moving rats. Brain Res Bull, 1984; 13: 39-42.

Kuret J, Schulman H. Purification and characterization of a Ca2+/calmodulin-dependent protein kinase from rat brain. Biochemistry, 1984; 23: 5495-504.

Lam BY, Zhang W, Enticknap N, Haggis E, Cader MZ, Chawla S. Inverse regulation of plasticity-related immediate early genes by calcineurin in hippocampal neurons. J Biol Chem, 2009.

Lang C, Barco A, Zablow L, Kandel ER, Siegelbaum SA, Zakharenko SS. Transient expansion of synaptically connected dendritic spines upon induction of hippocampal long-term potentiation. PNAS, 2004; 101: 16665-70.

Lee H, Barbarosie M, Kameyama K, Bear M, Huganir R. Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature, 2000; 405: 955-9.

Lee KS, Schottler F, Oliver M, Lynch G. Brief bursts of high-frequency stimulation produce two types of structural change in rat hippocampus. J Neurophysiol, 1980; 44: 247-58.

Leutgeb JK, Frey JU, Behnisch T. LTP in cultured hippocampal-entorhinal cortex slices from young adult (P25-30) rats. J Neurosci Methods, 2003; 130: 19-32.

Leutgeb S, Leutgeb JK, Treves A, Moser M-B, Moser EI. Distinct Ensemble Codes in Hippocampal Areas CA3 and CA1. Science, 2004; 305: 1295-8.

Levenson JM, O'Riordan KJ, Brown KD, Trinh MA, Molfese DL, Sweatt JD. Regulation of Histone Acetylation during Memory Formation in the Hippocampus. J Biol Chem, 2004; 279: 40545-59.

Li S, Cullen WK, Anwyl R, Rowan MJ. Dopamine-dependent facilitation of LTP induction in hippocampal CA1 by exposure to spatial novelty. Nat Neurosci, 2003; 6: 526-31.

Liao L, Pilotte J, Xu T, Wong CC, Edelman GM, Vanderklish P, Yates JR, 3rd. BDNF induces widespread changes in synaptic protein content and up-regulates components of the translation machinery: an analysis using high-throughput proteomics. J Proteome Res, 2007; 6: 1059-71.

Lin B, Kramar EA, Bi X, Brucher FA, Gall CM, Lynch G. Theta stimulation polymerizes actin in dendritic spines of hippocampus. J Neurosci, 2005; 25: 2062-9.

Ling DS, Benardo LS, Sacktor TC. Protein kinase Mzeta enhances excitatory synaptic transmission by increasing the number of active postsynaptic AMPA receptors. Hippocampus, 2006; 16: 443-52.

Ling DS, Benardo LS, Serrano PA, Blace N, Kelly MT, Crary JF, Sacktor TC. Protein kinase Mzeta is necessary and sufficient for LTP maintenance. Nat Neurosci, 2002; 5: 295-6.

Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: Evidence for membrane cycling from Golgi to ER. Cell, 1989; 56: 801-13.

Lipton P, Aitken PG, Dudek FE, Eskessen K, Espanol MT, Ferchmin PA, Kelly JB, Kreisman NR, Landfield PW, Larkman PM, Leybaert L, Newman GC, Panizzon KL, Payne RS, Phillips P, Raley-Susman KM, Rice ME, Santamaria R, Sarvey JM, Schurr A, Segal M, Sejer V, Taylor CP, Teyler TJ, Vasilenko VY, Veregge S, Wu SH, Wallis R. Making the best of brain slices; comparing preparative methods. Journal of Neuroscience Methods, 1995; 59: 151-6.

Lisman J, Raghavachari S. A Unified Model of the Presynaptic and Postsynaptic Changes During LTP at CA1 Synapses. Sci. STKE, 2006; 2006.

Lisman JE. A mechanism for the Hebb and the anti-Hebb processes underlying learning and memory. Proc Natl Acad Sci U S A, 1989; 86: 9574-8.

Lisman JE. Long-term potentiation: outstanding questions and attempted synthesis. Philos Trans R Soc Lond B Biol Sci, 2003; 358: 829-42.

Lisman JE, Goldring MA. Feasibility of long-term storage of graded information by the Ca2+/calmodulin-dependent protein kinase molecules of the postsynaptic density. Proc Natl Acad Sci U S A, 1988; 85: 5320-4.

Lisman JE, Grace AA. The Hippocampal-VTA Loop: Controlling the Entry of Information into Long-Term Memory. Neuron, 2005; 46: 703-13.

Lisman JE, Harris KM. Quantal analysis and synaptic anatomy--integrating two views of hippocampal plasticity. Trends Neurosci, 1993; 16: 141-7.

Lisman JE, Zhabotinsky AM. A model of synaptic memory: a CaMKII/PP1 switch that potentiates transmission by organizing an AMPA receptor anchoring assembly. Neuron, 2001; 31: 191-201.

Liu L, Wong T, Pozza M, Lingenhoehl K, Wang Y, Sheng M, Auberson Y, Wang Y. Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science, 2004; 304: 1021 - 4.

Lopez-Rojas J, Almaguer-Melian W, Bergado-Rosado JA. Synaptic tagging and memory trace. Rev Neurol, 2007; 45: 607-14.

Lopez-Salon M, Alonso M, Vianna MR, Viola H, Mello e Souza T, Izquierdo I, Pasquini JM, Medina JH. The ubiquitin-proteasome cascade is required for mammalian long-term memory formation. Eur J Neurosci, 2001; 14: 1820-6.

Luscher C, Nicoll RA, Malenka RC, Muller D. Synaptic plasticity and dynamic modulation of the postsynaptic membrane. Nat Neurosci, 2000; 3: 545-50.

Lushnikova I, Skibo G, Muller D, Nikonenko I. Synaptic potentiation induces increased glial coverage of excitatory synapses in CA1 hippocampus. Hippocampus, 2009; 19: 753-62.

Luthl A, Laurent JP, Figurov A, Muller D, Schachner M. Hippocampal long-term potentiation and neural cell adhesion molecules L1 and NCAM. Nature, 1994; 372: 777-9.

Lynch G, Rex CS, Gall CM. LTP consolidation: substrates, explanatory power, and functional significance. Neuropharmacology, 2007; 52: 12-23.

Lynch MA. Long-Term Potentiation and Memory. Physiol. Rev., 2004; 84: 87-136.

Lynch MA, Voss KL, Rodriguez J, Bliss TV. Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. Neuroscience, 1994; 60: 1-5.

Maas C, Belgardt D, Lee HK, Heisler FF, Lappe-Siefke C, Magiera MM, van Dijk J, Hausrat TJ, Janke C, Kneussel M. Synaptic activation modifies microtubules underlying transport of postsynaptic cargo. Proc Natl Acad Sci U S A, 2009; 106: 8731-6.

Madani R, Hulo S, Toni N, Madani H, Steimer T, Muller D, Vassalli JD. Enhanced hippocampal long-term potentiation and learning by increased neuronal expression of tissue-type plasminogen activator in transgenic mice. Embo J, 1999; 18: 3007-12.

Maggio N, Segal M. Differential modulation of long-term depression by acute stress in the rat dorsal and ventral hippocampus. J Neurosci, 2009; 29: 8633-8.

Majewska A, Tashiro A, Yuste R. Regulation of spine calcium dynamics by rapid spine motility. The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience, 2000; 20: 8262-8.

Makhinson M, Chotiner JK, Watson JB, O'Dell TJ. Adenylyl cyclase activation modulates activity-dependent changes in synaptic strength and Ca2+/calmodulin-dependent kinase II autophosphorylation. J Neurosci, 1999; 19: 2500-10.

Malenka RC, Kauer JA, Perkel DJ, Mauk MD, Kelly PT, Nicoll RA, Waxham MN. An essential role for postsynaptic calmodulin and protein kinase activity in long-term potentiation. Nature, 1989; 340: 554-7.

Maletic-Savatic M, Malinow R, Svoboda K. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science, 1999; 283: 1923-7.

Malinow R, Schulman H, Tsien RW. Inhibition of postsynaptic PKC or CaMKII blocks induction but not expression of LTP. Science, 1989; 245: 862-6.

Malleret G, Haditsch U, Genoux D, Jones MW, Bliss TV, Vanhoose AM, Weitlauf C, Kandel ER, Winder DG, Mansuy IM. Inducible and reversible enhancement of learning, memory, and long-term potentiation by genetic inhibition of calcineurin. Cell, 2001; 104: 675-86.

Manahan-Vaughan D, Braunewell KH. Novelty acquisition is associated with induction of hippocampal long-term depression. Proc Natl Acad Sci U S A, 1999; 96: 8739-44.

Manahan-Vaughan D, Reymann KG. Regional and developmental profile of modulation of hippocampal synaptic transmission and LTP by AP4-sensitive mGluRs in vivo. Neuropharmacology, 1995; 34: 991-1001.

Mandler G. Recognizing: The judgment of previous occurrence. Psychological Review, 1980; 87: 252-71.

Martin KC, Barad M, Kandel ER. Local protein synthesis and its role in synapse-specific plasticity. Curr Opin Neurobiol, 2000a; 10: 587-92.

Martin KC, Casadio A, Zhu H, Yaping E, Rose JC, Chen M, Bailey CH, Kandel ER. Synapse-specific, long-term facilitation of aplysia sensory to motor synapses: a function for local protein synthesis in memory storage. Cell, 1997; 91: 927-38.

Martin KC, Kosik KS. Synaptic tagging -- who's it? Nat Rev Neurosci, 2002; 3: 813-20.

Martin SJ, Grimwood PD, Morris RG. Synaptic plasticity and memory: an evaluation of the hypothesis. Annu Rev Neurosci, 2000b; 23: 649-711.

Martin SJ, Morris RG. New life in an old idea: the synaptic plasticity and memory hypothesis revisited. Hippocampus, 2002a; 12: 609-36.

Martin SJ, Morris RGM. New life in an old idea: the synaptic plasticity and memory hypothesis revisited. Hippocampus, 2002b; 12: 609-36.

Matsumoto-Miyai K, Ninomiya A, Yamasaki H, Tamura H, Nakamura Y, Shiosaka S. NMDA-Dependent Proteolysis of Presynaptic Adhesion Molecule L1 in the Hippocampus by Neuropsin. J. Neurosci., 2003; 23: 7727-36.

Matsuo N, Reijmers L, Mayford M. Spine-Type-Specific Recruitment of Newly Synthesized AMPA Receptors with Learning. Science, 2008; 319: 1104-7.

Matsuzaki M, Honkura N, Ellis-Davies GC, Kasai H. Structural basis of long-term potentiation in single dendritic spines. Nature, 2004; 429: 761-6.

Matthies H. In search of cellular mechanisms of memory. Progress in Neurobiology, 1989a; 32: 277-349.

Matthies H. Neurobiological Aspects of Learning and Memory. Annual Review of Psychology, 1989b; 40: 381-404.

Mayevsky A, Zeuthen T, Chance B. Measurements of extracellular potassium, ECoG and pyridine nucleotide levels during cortical spreading depression in rats. Brain Research, 1974; 76: 347-9.

Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER. Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in Aplysia. Science, 1992; 256: 638-44.

McGaugh JL. Memory--a century of consolidation. Science, 2000; 287: 248-51.

McGlade-McCulloh E, Yamamoto H, Tan SE, Brickey DA, Soderling TR. Phosphorylation and regulation of glutamate receptors by calcium/calmodulin-dependent protein kinase II. Nature, 1993; 362: 640-2.

McIntyre CK, Miyashita T, Setlow B, Marjon KD, Steward O, Guzowski JF, McGaugh JL. Memory-influencing intra-basolateral amygdala drug infusions modulate expression of Arc protein in the hippocampus. Proc Natl Acad Sci U S A, 2005.

Meeter M, Murre JM, Talamini LM. Mode shifting between storage and recall based on novelty detection in oscillating hippocampal circuits. Hippocampus, 2004; 14: 722-41.

Meshul CK, Hopkins WF. Presynaptic ultrastructural correlates of long-term potentiation in the CA1 subfield of the hippocampus. Brain Res, 1990; 514: 310-9.

Messaoudi E, Kanhema T, Soule J, Tiron A, Dagyte G, da Silva B, Bramham CR. Sustained Arc/Arg3.1 synthesis controls long-term potentiation consolidation through regulation of local actin polymerization in the dentate gyrus in vivo. J Neurosci, 2007; 27: 10445-55.

Messaoudi E, Ying SW, Kanhema T, Croll SD, Bramham CR. Brain-derived neurotrophic factor triggers transcription-dependent, late phase long-term potentiation in vivo. J Neurosci, 2002; 22: 7453-61.

Meyer T, Hanson PI, Stryer L, Schulman H. Calmodulin trapping by calcium-calmodulin-dependent protein kinase. Science, 1992; 256: 1199-202.

Migaud M, Charlesworth P, Dempster M, Webster LC, Watabe AM, Makhinson M, He Y, Ramsay MF, Morris RG, Morrison JH, O'Dell TJ, Grant SG. Enhanced long-term potentiation and impaired learning in mice with mutant postsynaptic density-95 protein. Nature, 1998; 396: 433-9.

Miles R, Poncer JC, Fricker D, Leinekugel X. The birth (and adolescence) of LTP. J Physiol, 2005; 568: 1-2.

Miller B, Sarantis M, Traynelis SF, Attwell D. Potentiation of NMDA receptor currents by arachidonic acid. Nature, 1992; 355: 722-5.

Miller P, Zhabotinsky AM, Lisman JE, Wang XJ. The stability of a stochastic CaMKII switch: dependence on the number of enzyme molecules and protein turnover. PLoS Biol, 2005; 3: e107.

Mirenowicz J, Schultz W. Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature, 1996; 379: 449-51.

Miyashita T, Kubik S, Haghighi N, Steward O, Guzowski JF. Rapid activation of plasticity-associated gene transcription in hippocampal neurons provides a mechanism for encoding of one-trial experience. J Neurosci, 2009; 29: 898-906.

Mizutani A, Saito H, Matsuki N. Possible involvement of plasmin in long-term potentiation of rat hippocampal slices. Brain Res, 1996; 739: 276-81.

Mizutani A, Tanaka T, Saito H, Matsuki N. Postsynaptic blockade of inhibitory postsynaptic currents by plasmin in CA1 pyramidal cells of rat hippocampus. Brain Res, 1997; 761: 93-6.

Moccia R, Chen D, Lyles V, Kapuya E, E Y, Kalachikov S, Spahn CMT, Frank J, Kandel ER, Barad et a. An unbiased cDNA library prepared from isolated Aplysia sensory neuron processes is enriched for cytoskeletal and translational mRNAs. The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience, 2003; 23: 9409-17.

Mockett BG, Brooks WM, Tate WP, Abraham WC. Dopamine D1/D5 receptor activation fails to initiate an activity-independent late-phase LTP in rat hippocampus. Brain Res, 2004; 1021: 92-100.

Moncada D, Viola H. Induction of Long-Term Memory by Exposure to Novelty Requires Protein Synthesis: Evidence for a Behavioral Tagging. J. Neurosci., 2007; 27: 7476-81.

Morozov A, Muzzio IA, Bourtchouladze R, Van-Strien N, Lapidus K, Yin D, Winder DG, Adams JP, Sweatt JD, Kandel ER. Rap1 couples cAMP signaling to a distinct pool of p42/44MAPK regulating excitability, synaptic plasticity, learning, and memory. Neuron, 2003; 39: 309-25.

Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods, 1984; 11: 47-60.

Morris RG, Garrud P, Rawlins JN, O'Keefe J. Place navigation impaired in rats with hippocampal lesions. Nature, 1982; 297: 681-3.

Morris RG, Moser EI, Riedel G, Martin SJ, Sandin J, Day M, O'Carroll C. Elements of a neurobiological theory of the hippocampus: the role of activity-dependent synaptic plasticity in memory. Philos Trans R Soc Lond B Biol Sci, 2003; 358: 773-86.

Morris RGM, Anderson E, Lynch GS, Baudry M. Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. 1986; 319: 774-6.

Moser E, Mathiesen I, Andersen P. Association between brain temperature and dentate field potentials in exploring and swimming rats. Science, 1993; 259: 1324-6.

Muller D, Wang C, Skibo G, Toni N, Cremer H, Calaora V, Rougon G, Kiss JZ. PSA-NCAM is required for activity-induced synaptic plasticity. Neuron, 1996; 17: 413-22.

Nagy V, Bozdagi O, Matynia A, Balcerzyk M, Okulski P, Dzwonek J, Costa RM, Silva AJ, Kaczmarek L, Huntley GW. Matrix metalloproteinase-9 is required for hippocampal late-phase long-term potentiation and memory. J Neurosci, 2006; 26: 1923-34.

Naisbitt S, Kim E, Tu JC, Xiao B, Sala C, Valtschanoff J, Weinberg RJ, Worley PF, Sheng M. Shank, a novel family of postsynaptic density proteins that binds to the NMDA receptor/PSD-95/GKAP complex and cortactin. Neuron, 1999; 23: 569-82.

Nakagami Y, Abe K, Nishiyama N, Matsuki N. Laminin degradation by plasmin regulates long-term potentiation. J Neurosci, 2000; 20: 2003-10.

Nakazawa K, Sun LD, Quirk MC, Rondi-Reig L, Wilson MA, Tonegawa S. Hippocampal CA3 NMDA receptors are crucial for memory acquisition of one-time experience. Neuron, 2003; 38: 305-15.

Navakkode S, Sajikumar S, Frey JU. Mitogen-activated protein kinase-mediated reinforcement of hippocampal early long-term depression by the type IV-specific phosphodiesterase inhibitor rolipram and its effect on synaptic tagging. J Neurosci, 2005; 25: 10664-70.

Navakkode S, Sajikumar S, Frey JU. Synergistic requirements for the induction of dopaminergic D1/D5-receptor-mediated LTP in hippocampal slices of rat CA1 in vitro. Neuropharmacology, 2007; 52: 1547-54.

Navakkode S, Sajikumar S, Frey JU. The type IV-specific phosphodiesterase inhibitor rolipram and its effect on hippocampal long-term potentiation and synaptic tagging. J Neurosci, 2004; 24: 7740-4.

Nayak A, Zastrow DJ, Lickteig R, Zahniser NR, Browning MD. Maintenance of late-phase LTP is accompanied by PKA-dependent increase in AMPA receptor synthesis. Nature, 1998; 394: 680-3.

Nguyen PV, Abel T, Kandel ER. Requirement of a critical period of transcription for induction of a late phase of LTP. Science, 1994; 265: 1104-7.

Nguyen PV, Woo NH. Regulation of hippocampal synaptic plasticity by cyclic AMP-dependent protein kinases. Progress in Neurobiology, 2003; 71: 401-37.

Nicoll RA, Alger BE. A simple chamber for recording from submerged brain slices. J Neurosci Methods, 1981; 4: 153-6.

Nie T, McDonough CB, Huang T, Nguyen PV, Abel T. Genetic disruption of protein kinase A anchoring reveals a role for compartmentalized kinase signaling in theta-burst long-term potentiation and spatial memory. J Neurosci, 2007; 27: 10278-88.

O'Carroll CM, Martin SJ, Sandin J, Frenguelli B, Morris RGM. Dopaminergic modulation of the persistence of one-trial hippocampus-dependent memory. Learn. Mem., 2006; 13: 760-9.

O'Carroll CM, Morris RGM. Heterosynaptic co-activation of glutamatergic and dopaminergic afferents is required to induce persistent long-term potentiation. Neuropharmacology, 2004; 47: 324-32.

O'Keefe J, Dostrovsky J. The hippocampus as a spatial map. Preliminary evidence from unit activity in the freely-moving rat. Brain Res, 1971; 34: 171-5.

O'Reilly RC, Rudy JW. Computational principles of learning in the neocortex and hippocampus. Hippocampus, 2000; 10: 389-97.

Oh JS, Manzerra P, Kennedy MB. Regulation of the neuron-specific Ras GTPase-activating protein, synGAP, by Ca2+/calmodulin-dependent protein kinase II. J Biol Chem, 2004; 279: 17980-8.

Okada D, Ozawa F, Inokuchi K. Input-Specific Spine Entry of Soma-Derived Vesl-1S Protein Conforms to Synaptic Tagging. Science, 2009; 324: 904-9.

Okada D, Yamagishi S, Sugiyama H. Differential effects of phospholipase inhibitors in long-term potentiation in the rat hippocampal mossy fiber synapses and Schaffer/commissural synapses. Neurosci Lett, 1989; 100: 141-6.

Okamoto K, Nagai T, Miyawaki A, Hayashi Y. Rapid and persistent modulation of actin dynamics regulates postsynaptic reorganization underlying bidirectional plasticity. Nat Neurosci, 2004; 7: 1104-12.

Okamura K, Tanaka H, Yagita Y, Saeki Y, Taguchi A, Hiraoka Y, Zeng L-H, R Colman D, Miki N. Cadherin activity is required for activity-induced spine remodeling
10.1083/jcb.200406030. J. Cell Biol., 2004; 167: 961-72.

Osten P, Valsamis L, Harris A, Sacktor TC. Protein synthesis-dependent formation of protein kinase Mzeta in long-term potentiation. J Neurosci, 1996; 16: 2444-51.

Ostroff LE, Fiala JC, Allwardt B, Harris KM. Polyribosomes redistribute from dendritic shafts into spines with enlarged synapses during LTP in developing rat hippocampal slices. Neuron, 2002; 35: 535-45.

Otmakhov N, Griffith LC, Lisman JE. Postsynaptic Inhibitors of Calcium/Calmodulin-Dependent Protein Kinase Type II Block Induction But Not Maintenance of Pairing-Induced Long-Term Potentiation. J. Neurosci., 1997; 17: 5357-65.

Otmakhov N, Khibnik L, Otmakhova N, Carpenter S, Riahi S, Asrican B, Lisman J. Forskolin-induced LTP in the CA1 hippocampal region is NMDA receptor dependent. J Neurophysiol, 2004; 91: 1955-62.

Otmakhova NA, Lisman JE. D1/D5 Dopamine Receptor Activation Increases the Magnitude of Early Long-Term Potentiation at CA1 Hippocampal Synapses. J. Neurosci., 1996; 16: 7478-86.

Ouyang Y, Kantor D, Harris KM, Schuman EM, Kennedy MB. Visualization of the distribution of autophosphorylated calcium/calmodulin-dependent protein

kinase II after tetanic stimulation in the CA1 area of the hippocampus. J Neurosci, 1997; 17: 5416-27.

Pak DT, Sheng M. Targeted protein degradation and synapse remodeling by an inducible protein kinase. Science, 2003; 302: 1368-73. Epub 2003 Oct 23.

Pak DT, Yang S, Rudolph-Correia S, Kim E, Sheng M. Regulation of dendritic spine morphology by SPAR, a PSD-95-associated RapGAP. Neuron, 2001; 31: 289-303.

Pang PT, Lu B. Regulation of late-phase LTP and long-term memory in normal and aging hippocampus: role of secreted proteins tPA and BDNF. Ageing Res Rev, 2004; 3: 407-30.

Pantaloni D, Le Clainche C, Carlier MF. Mechanism of actin-based motility. Science, 2001; 292: 1502-6.

Park CS, Gong R, Stuart J, Tang S-J. Molecular Network and Chromosomal Clustering of Genes Involved in Synaptic Plasticity in the Hippocampus. J. Biol. Chem., 2006; 281: 30195-211.

Pastalkova E, Serrano P, Pinkhasova D, Wallace E, Fenton AA, Sacktor TC. Storage of Spatial Information by the Maintenance Mechanism of LTP 10.1126/science.1128657. Science, 2006; 313: 1141-4.

Pavlov I, Voikar V, Kaksonen M, Lauri SE, Hienola A, Taira T, Rauvala H. Role of heparin-binding growth-associated molecule (HB-GAM) in hippocampal LTP and spatial learning revealed by studies on overexpressing and knockout mice. Mol Cell Neurosci, 2002; 20: 330-42.

Pilpel Y, Segal M. Activation of PKC induces rapid morphological plasticity in dendrites of hippocampal neurons via Rac and Rho-dependent mechanisms. Eur J Neurosci, 2004; 19: 3151-64.

Poncer JC. Hippocampal long term potentiation: silent synapses and beyond. Journal of Physiology-Paris
Neuroscience and Computation, 2003; 97: 415-22.

Poon M, Choi S, Jamieson C, Geschwind D, Martin K. Identification of process-localized mRNAs from cultured rodent hippocampal neurons. J Neurosci, 2006; 26: 13390-9.

Qian Z, Gilbert ME, Colicos MA, Kandel ER, Kuhl D. Tissue-plasminogen activator is induced as an immediate-early gene during seizure, kindling and long-term potentiation. Nature, 1993; 361: 453-7.

Rabenstein R, Addy N, Caldarone B, Asaka Y, Gruenbaum L, Peters L, Gilligan D, Fitzsimonds R, Picciotto M. Impaired synaptic plasticity and learning in mice lacking beta-adducin, an actin-regulating protein., 2005; 25: -.

Raymond CR. Different requirements for action potentials in the induction of different forms of long-term potentiation. J Physiol, 2008; 586: 1859-65.

Reid KH, Edmonds HL, Jr., Schurr A, Tseng MT, West CA. Pitfalls in the use of brain slices. Prog Neurobiol, 1988; 31: 1-18.

Reist C, Duffy JG, Fujimoto K, Cahill L. beta-Adrenergic blockade and emotional memory in PTSD. Int J Neuropsychopharmacol, 2001; 4: 377-83.

Rempel-Clower NL, Zola SM, Squire LR, Amaral DG. Three cases of enduring memory impairment after bilateral damage limited to the hippocampal formation. J Neurosci, 1996; 16: 5233-55.

Remy S, Spruston N. Dendritic spikes induce single-burst long-term potentiation. Proc Natl Acad Sci U S A, 2007; 104: 17192-7.

Rex CS, Lin C-Y, Kramar EA, Chen LY, Gall CM, Lynch G. Brain-Derived Neurotrophic Factor Promotes Long-Term Potentiation-Related Cytoskeletal Changes in Adult Hippocampus. J. Neurosci., 2007; 27: 3017-29.

Reymann KG, Frey JU. The late maintenance of hippocampal LTP: Requirements, phases, `synaptic tagging', `late-associativity' and implications. Neuropharmacology, 2007; 52: 24-40.

Reymann KG, Malisch R, Schulzeck K, Brodemann R, Ott T, Matthies H. The duration of long-term potentiation in the CA1 region of the hippocampal slice preparation. Brain Res Bull, 1985; 15: 249-55.

Rial Verde EM, Lee-Osbourne J, Worley Pn-bsF, Malinow R, Cline Hn-bsT. Increased Expression of the Immediate-Early Gene Arc/Arg3.1 Reduces AMPA Receptor-Mediated Synaptic Transmission. Neuron, 2006; 52: 461-74.

Richter JD, Klann E. Making synaptic plasticity and memory last: mechanisms of translational regulation. Genes & development, 2009; 23: 1-11.

Rizo J, Sudhof TC. Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci, 2002; 3: 641-53.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD. The Mitogen-Activated Protein Kinase Cascade Couples PKA and PKC to cAMP Response Element Binding Protein Phosphorylation in Area CA1 of Hippocampus. J. Neurosci., 1999; 19: 4337-48.

Rose SP. Cell-adhesion molecules, glucocorticoids and long-term-memory formation. Trends Neurosci, 1995; 18: 502-6.

Routtenberg A, Rekart JL. Post-translational protein modification as the substrate for long-lasting memory. Trends Neurosci, 2005; 28: 12-9.

Rudy JW, Sutherland RJ. Configural association theory and the hippocampal formation: an appraisal and reconfiguration. Hippocampus, 1995; 5: 375-89.

Rumbaugh G, Adams JP, Kim JH, Huganir RL. SynGAP regulates synaptic strength and mitogen-activated protein kinases in cultured neurons. Proc Natl Acad Sci U S A, 2006; 103: 4344-51.

Sabatini BL, Maravall M, Svoboda K. Ca2+ signaling in dendritic spines. Current Opinion in Neurobiology, 2001; 11: 349-56.

Sajikumar S, Frey JU. Late-associativity, synaptic tagging, and the role of dopamine during LTP and LTD. Neurobiol Learn Mem, 2004a; 82: 12-25.

Sajikumar S, Frey JU. Resetting of 'synaptic tags' is time- and activity-dependent in rat hippocampal CA1 in vitro. Neuroscience, 2004b; 129: 503-7.

Sajikumar S, Navakkode S, Frey JU. Distinct single but not necessarily repeated tetanization is required to induce hippocampal late-LTP in the rat CA1. Learn. Mem., 2008; 15: 46-9.

Sajikumar S, Navakkode S, Frey JU. Identification of Compartment- and Process-Specific Molecules Required for "Synaptic Tagging" during Long-Term Potentiation and Long-Term Depression in Hippocampal CA1. J. Neurosci., 2007; 27: 5068-80.

Sajikumar S, Navakkode S, Frey JU. Protein synthesis-dependent long-term functional plasticity: methods and techniques. Current Opinion in Neurobiology Neuronal and glial cell biology / New technologies, 2005a; 15: 607-13.

Sajikumar S, Navakkode S, Frey JU. Protein synthesis-dependent long-term functional plasticity: methods and techniques. Curr Opin Neurobiol, 2005b; 15: 607-13.

Sajikumar S, Navakkode S, Sacktor TC, Frey JU. Synaptic tagging and cross-tagging: the role of protein kinase Mzeta in maintaining long-term potentiation but not long-term depression. J Neurosci, 2005c; 25: 5750-6.

Sala C, Piech V, Wilson NR, Passafaro M, Liu G, Sheng M. Regulation of dendritic spine morphology and synaptic function by Shank and Homer. Neuron, 2001; 31: 115-30.

Sandi C, Loscertales M, Guaza C. Experience-dependent facilitating effect of corticosterone on spatial memory formation in the water maze. Eur J Neurosci, 1997; 9: 637-42.

Sanhueza M, McIntyre CC, Lisman JE. Reversal of synaptic memory by Ca2+/calmodulin-dependent protein kinase II inhibitor. J Neurosci, 2007; 27: 5190-9.

Sato M, Suzuki K, Nakanishi S. NMDA receptor stimulation and brain-derived neurotrophic factor upregulate homer 1a mRNA via the mitogen-activated protein kinase cascade in cultured cerebellar granule cells. J Neurosci, 2001; 21: 3797-805.

Scharf MT, Woo NH, Lattal KM, Young JZ, Nguyen PV, Abel T. Protein synthesis is required for the enhancement of long-term potentiation and long-term memory by spaced training. J Neurophysiol, 2002; 87: 2770-7.

Scheetz AJ, Nairn AC, Constantine-Paton M. NMDA receptor-mediated control of protein synthesis at developing synapses. Nat Neurosci, 2000; 3: 211-6.

Schmitt JM, Guire ES, Saneyoshi T, Soderling TR. Calmodulin-dependent kinase kinase/calmodulin kinase I activity gates extracellular-regulated kinase-dependent long-term potentiation. J Neurosci, 2005; 25: 1281-90.

Schuchmann S, Meierkord H, Stenkamp K, Breustedt J, Windmuller O, Heinemann U, Buchheim K. Synaptic and nonsynaptic ictogenesis occurs at different temperatures in submerged and interface rat brain slices. J Neurophysiol, 2002; 87: 2929-35.

Schultz W. Multiple reward signals in the brain. Nat Rev Neurosci, 2000; 1: 199-207.

Schuman EM, Dynes JL, Steward O. Synaptic Regulation of Translation of Dendritic mRNAs. J. Neurosci., 2006; 26: 7143-6.

Schurr A, Reid KH, Tseng MT, Edmonds HL, Jr., West CA, Rigor BM. Effect of electrical stimulation on the viability of the hippocampal slice preparation. Brain Res Bull, 1986; 16: 299-301.

Serrano P, Yao Y, Sacktor TC. Persistent phosphorylation by protein kinase Mzeta maintains late-phase long-term potentiation. J Neurosci, 2005; 25: 1979-84.

Sharma K, Fong DK, Craig AM. Postsynaptic protein mobility in dendritic spines: long-term regulation by synaptic NMDA receptor activation. Mol Cell Neurosci, 2006; 31: 702-12.

Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annual review of biochemistry, 1999; 68: 821-61.

Shen H, Watanabe M, Tomasiewicz H, Rutishauser U, Magnuson T, Glass JD. Role of Neural Cell Adhesion Molecule and Polysialic Acid in Mouse Circadian Clock Function. J. Neurosci., 1997; 17: 5221-9.

Shen K, Meyer T. Dynamic control of CaMKII translocation and localization in hippocampal neurons by NMDA receptor stimulation. Science, 1999; 284: 162-6.

Shepherd JD, Rumbaugh G, Wu J, Chowdhury S, Plath N, Kuhl D, Huganir RL, Worley PF. Arc/Arg3.1 Mediates Homeostatic Synaptic Scaling of AMPA Receptors. Neuron, 2006; 52: 475-84.

Shukla K, Kim J, Blundell J, Powell CM. Learning-induced Glutamate Receptor Phosphorylation Resembles That Induced by Long Term Potentiation 10.1074/jbc.M702906200. J. Biol. Chem., 2007; 282: 18100-7.

Si K, Giustetto M, Etkin A, Hsu R, Janisiewicz AM, Miniaci MC, Kim JH, Zhu H, Kandel ER. A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell, 2003a; 115: 893-904.

Si K, Lindquist S, Kandel ER. A neuronal isoform of the aplysia CPEB has prion-like properties. Cell, 2003b; 115: 879-91.

Silva AJ, Stevens CF, Tonegawa S, Wang Y. Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science, 1992; 257: 201-6.

Silverman ES, Du J, Williams AJ, Wadgaonkar R, Drazen JM, Collins T. cAMP-response-element-binding-protein-binding protein (CBP) and p300 are transcriptional co-activators of early growth response factor-1 (Egr-1). Biochem J, 1998; 336 (Pt 1): 183-9.

Skrede KK, Westgaard RH. The transverse hippocampal slice: a well-defined cortical structure maintained in vitro. Brain Res, 1971; 35: 589-93.

Smolen P. A model of late long-term potentiation simulates aspects of memory maintenance. PLoS ONE, 2007; 2: e445.

Sokolov MV, Rossokhin AV, Astrelin AV, Frey JU, Voronin LL. Quantal analysis suggests strong involvement of presynaptic mechanisms during the initial 3 h maintenance of long-term potentiation in rat hippocampal CA1 area in vitro. Brain Research, 2002; 957: 61-75.

Song B, Meng F, Yan X, Guo J, Zhang G. Cerebral ischemia immediately increases serine phosphorylation of the synaptic RAS-GTPase activating protein SynGAP by calcium/calmodulin-dependent protein kinase II alpha in hippocampus of rats. Neurosci Lett, 2003; 349: 183-6.

Song I, Huganir RL. Regulation of AMPA receptors during synaptic plasticity. Trends in Neurosciences, 2002; 25: 578-88.

Sorra KE, Harris KM. Stability in synapse number and size at 2 hr after long-term potentiation in hippocampal area CA1. J Neurosci, 1998; 18: 658-71.

Spacek J, Harris KM. Three-dimensional organization of smooth endoplasmic reticulum in hippocampal CA1 dendrites and dendritic spines of the immature and mature rat. J Neurosci, 1997; 17: 190-203.

Squire LR. Memory and the hippocampus: a synthesis from findings with rats, monkeys, and humans. Psychol Rev, 1992; 99: 195-231.

Squire LR. Memory systems of the brain: a brief history and current perspective. Neurobiol Learn Mem, 2004; 82: 171-7.

Stanton PK, Sarvey JM. Blockade of long-term potentiation in rat hippocampal CA1 region by inhibitors of protein synthesis. J Neurosci, 1984; 4: 3080-8.

Stanton PK, Sejnowski TJ. Associative long-term depression in the hippocampus induced by hebbian covariance. Nature, 1989; 339: 215-8.

Staubli U, Chun D. Factors regulating the reversibility of long-term potentiation. J Neurosci, 1996; 16: 853-60.

Staubli U, Chun D, Lynch G. Time-Dependent Reversal of Long-Term Potentiation by an Integrin Antagonist. J. Neurosci., 1998; 18: 3460-9.

Steele RJ, Morris RG. Delay-dependent impairment of a matching-to-place task with chronic and intrahippocampal infusion of the NMDA-antagonist D-AP5. Hippocampus, 1999; 9: 118-36.

Steward O. mRNA localization in neurons: a multipurpose mechanism? Neuron, 1997; 18: 9-12.

Steward O, Fass B. Polyribosomes associated with dendritic spines in the denervated dentate gyrus: evidence for local regulation of protein synthesis during reinnervation. Progress in brain research, 1983; 58: 131-6.

Steward O, Schuman EM. Compartmentalized synthesis and degradation of proteins in neurons. Neuron, 2003; 40: 347-59.

Steward O, Schuman EM. Protein synthesis at synaptic sites on dendrites. Annu Rev Neurosci, 2001; 24: 299-325.

Steward O, Wallace CS, Lyford GL, Worley PF. Synaptic Activation Causes the mRNA for the IEG Arc to Localize Selectively near Activated Postsynaptic Sites on Dendrites. Neuron, 1998; 21: 741-51.

Steward O, Worley PF. Selective Targeting of Newly Synthesized Arc mRNA to Active Synapses Requires NMDA Receptor Activation. Neuron, 2001; 30: 227-40.

Strack S, Barban MA, Wadzinski BE, Colbran RJ. Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. J Neurochem, 1997a; 68: 2119-28.

Strack S, Choi S, Lovinger DM, Colbran RJ. Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. J Biol Chem, 1997b; 272: 13467-70.

Straube T, Korz V, Balschun D, Frey JU. Requirement of beta-adrenergic receptor activation and protein synthesis for LTP-reinforcement by novelty in rat dentate gyrus. J Physiol, 2003; 552: 953-60.

Sumi M, Kiuchi K, Ishikawa T, Ishii A, Hagiwara M, Nagatsu T, Hidaka H. The newly synthesized selective Ca2+/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. Biochem Biophys Res Commun, 1991; 181: 968-75.

Svoboda K, Tank DW, Denk W. Direct measurement of coupling between dendritic spines and shafts. Science, 1996; 272: 716-9.

Swanson-Park JL, Coussens CM, Mason-Parker SE, Raymond CR, Hargreaves EL, Dragunow M, Cohen AS, Abraham WC. A double dissociation within the hippocampus of dopamine D1/D5 receptor and beta-adrenergic receptor contributions to the persistence of long-term potentiation. Neuroscience, 1999; 92: 485-97.

Sweatt JD. Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol, 2004; 14: 311-7.

Tai CY, Mysore SP, Chiu C, Schuman EM. Activity-regulated N-cadherin endocytosis. Neuron, 2007; 54: 771-85.

Takemoto-Kimura S, Ageta-Ishihara N, Nonaka M, Adachi-Morishima A, Mano T, Okamura M, Fujii H, Fuse T, Hoshino M, Suzuki S, Kojima M, Mishina M, Okuno H, Bito H. Regulation of dendritogenesis via a lipid-raft-associated Ca2+/calmodulin-dependent protein kinase CLICK-III/CaMKIgamma. Neuron, 2007; 54: 755-70.

Talarico JM, Rubin DC. Confidence, not consistency, characterizes flashbulb memories. Psychological Science, 2003; 14: 455-61.

Tamura H, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S, Shiosaka S. Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. J Physiol, 2006a; 570: 541-51.

Tamura H, Ishikawa Y, Hino N, Maeda M, Yoshida S, Kaku S, Shiosaka S. Neuropsin is essential for early processes of memory acquisition and Schaffer collateral long-term potentiation in adult mouse hippocampus in vivo. J Physiol, 2006b; 570: 541-51.

Tanaka J-i, Horiike Y, Matsuzaki M, Miyazaki T, Ellis-Davies GCR, Kasai H. Protein Synthesis and Neurotrophin-Dependent Structural Plasticity of Single Dendritic Spines. Science, 2008; 319: 1683-7.

Taqatqeh F, Mergia E, Neitz A, Eysel UT, Koesling D, Mittmann T. More than a retrograde messenger: nitric oxide needs two cGMP pathways to induce hippocampal long-term potentiation. J Neurosci, 2009; 29: 9344-50.

Theis M, Si K, Kandel ER. Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus. Proceedings Of The National Academy Of Sciences Of The United States Of America, 2003; 100: 9602-7.

Thiel G, Schoch S, Petersohn D. Regulation of synapsin I gene expression by the zinc finger transcription factor zif268/egr-1. J Biol Chem, 1994; 269: 15294-301.

Thomas MJ, Moody TD, Makhinson M, O'Dell TJ. Activity-dependent beta-adrenergic modulation of low frequency stimulation induced LTP in the hippocampal CA1 region. Neuron, 1996; 17: 475-82.

Tokumitsu H, Inuzuka H, Ishikawa Y, Ikeda M, Saji I, Kobayashi R. STO-609, a specific inhibitor of the Ca(2+)/calmodulin-dependent protein kinase kinase. J Biol Chem, 2002; 277: 15813-8.

Tomita S, Stein V, Stocker TJ, Nicoll RA, Bredt DS. Bidirectional synaptic plasticity regulated by phosphorylation of stargazin-like TARPs. Neuron, 2005; 45: 269-77.

Tsokas P, Grace EA, Chan P, Ma T, Sealfon SC, Iyengar R, Landau EM, Blitzer RD. Local Protein Synthesis Mediates a Rapid Increase in Dendritic Elongation Factor 1A after Induction of Late Long-Term Potentiation. J. Neurosci., 2005; 25: 5833-43.

Tu JC, Xiao B, Naisbitt S, Yuan JP, Petralia RS, Brakeman P, Doan A, Aakalu VK, Lanahan AA, Sheng M, Worley PF. Coupling of mGluR/Homer and PSD-95 complexes by the Shank family of postsynaptic density proteins. Neuron, 1999; 23: 583-92.

Tully T. Toward a molecular biology of memory: the light's coming on! Nat Neurosci, 1998; 1: 543-5.

Turner BM. Cellular memory and the histone code. Cell, 2002; 111: 285-91.

Tzingounis AV, Nicoll RA. Arc/Arg3.1: Linking Gene Expression to Synaptic Plasticity and Memory. Neuron, 2006; 52: 403-7.

Urrutia R, Henley JR, Cook T, McNiven MA. The dynamins: redundant or distinct functions for an expanding family of related GTPases? Proc Natl Acad Sci U S A, 1997; 94: 377-84.

Vankov A, Herve-Minvielle A, Sara SJ. Response to novelty and its rapid habituation in locus coeruleus neurons of the freely exploring rat. Eur J Neurosci, 1995; 7: 1180-7.

Vazdarjanova A, Guzowski JF. Differences in hippocampal neuronal population responses to modifications of an environmental context: evidence for distinct, yet complementary, functions of CA3 and CA1 ensembles. J Neurosci, 2004; 24: 6489-96.

Vazdarjanova A, McNaughton BL, Barnes CA, Worley PF, Guzowski JF. Experience-Dependent Coincident Expression of the Effector Immediate-Early Genes Arc and Homer 1a in Hippocampal and Neocortical Neuronal Networks. J. Neurosci., 2002; 22: 10067-71.

Vazdarjanova A, Ramirez-Amaya V, Insel N, Plummer TK, Rosi S, Chowdhury S, Mikhael D, Worley PF, Guzowski JF, Barnes CA. Spatial exploration induces ARC, a plasticity-related immediate-early gene, only in calcium/calmodulin-dependent protein kinase II-positive principal excitatory and inhibitory neurons of the rat forebrain. J Comp Neurol, 2006; 498: 317-29.

Vickers CA, Dickson KS, Wyllie DJ. Induction and maintenance of late-phase long-term potentiation in isolated dendrites of rat hippocampal CA1 pyramidal neurones. J Physiol, 2005.

Vickers CA, Wyllie DJA. Late-phase, protein synthesis-dependent long-term potentiation in hippocampal CA1 pyramidal neurones with destabilized microtubule networks. 2007.

Viosca J, Jancic D, Lopez-Atalaya JP, Benito E. Hunting for Synaptic Tagging and Capture in Memory Formation. J. Neurosci., 2007; 27: 12761-3.

Wagatsuma H, Yamaguchi Y. Neural dynamics of the cognitive map in the hippocampus. Cogn Neurodyn, 2007; 1: 119-41.

Walikonis RS, Oguni A, Khorosheva EM, Jeng CJ, Asuncion FJ, Kennedy MB. Densin-180 forms a ternary complex with the (alpha)-subunit of Ca2+/calmodulin-dependent protein kinase II and (alpha)-actinin. J Neurosci, 2001; 21: 423-33.

Wang H, Gerkin R, Nauen D, Bi G. Coactivation and timing-dependent integration of synaptic potentiation and depression. Nat Neurosci, 2005; 8: 187 - 93.

Wang Z, Edwards JG, Riley N, Provance DW, Jr., Karcher R, Li XD, Davison IG, Ikebe M, Mercer JA, Kauer JA, Ehlers MD. Myosin Vb mobilizes recycling endosomes and AMPA receptors for postsynaptic plasticity. Cell, 2008; 135: 535-48.

Watabe AM, Zaki PA, O'Dell TJ. Coactivation of beta-adrenergic and cholinergic receptors enhances the induction of long-term potentiation and synergistically activates mitogen-activated protein kinase in the hippocampal CA1 region. J Neurosci, 2000; 20: 5924-31.

Watson GB, Lopez OT, Charles VD, Lanthorn TH. Assessment of long-term effects of transient anoxia on metabolic activity of rat hippocampal slices using triphenyltetrazolium chloride. Journal of Neuroscience Methods, 1994; 53: 203-8.

Watson PL, Weiner JL, Carlen PL. Effects of variations in hippocampal slice preparation protocol on the electrophysiological stability, epileptogenicity and graded hypoxia responses of CA1 neurons. Brain Res, 1997; 775: 134-43.

Whitlock JR, Heynen AJ, Shuler MG, Bear MF. Learning Induces Long-Term Potentiation in the Hippocampus. Science, 2006; 313: 1093-7.

Wickham L, Duchaine T, Luo M, Nabi IR, DesGroseillers L. Mammalian staufen is a double-stranded-RNA- and tubulin-binding protein which localizes to the rough endoplasmic reticulum. Molecular and cellular biology, 1999; 19: 2220-30.

Wigstrom H, Gustafsson B. Postsynaptic control of hippocampal long-term potentiation. Journal de physiologie, 1986; 81: 228-36.

Williams JH, Errington ML, Lynch MA, Bliss TV. Arachidonic acid induces a long-term activity-dependent enhancement of synaptic transmission in the hippocampus. Nature, 1989; 341: 739-42.

Woo NH, Abel T, Nguyen PV. Genetic and pharmacological demonstration of a role for cyclic AMP-dependent protein kinase-mediated suppression of protein phosphatases in gating the expression of late LTP. Eur J Neurosci, 2002; 16: 1871-6.

Wood MA, Kaplan MP, Park A, Blanchard EJ, Oliveira AM, Lombardi TL, Abel T. Transgenic mice expressing a truncated form of CREB-binding protein (CBP) exhibit deficits in hippocampal synaptic plasticity and memory storage. Learn Mem, 2005; 12: 111-9.

Wu GY, Deisseroth K, Tsien RW. Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A, 2001; 98: 2808-13.

Xiao B, Tu JC, Worley PF. Homer: a link between neural activity and glutamate receptor function. Curr Opin Neurobiol, 2000; 10: 370-4.

Xiao MY, Karpefors M, Gustafsson B, Wigstrom H. On the linkage between AMPA and NMDA receptor-mediated EPSPs in homosynaptic long-term depression in the hippocampal CA1 region of young rats. J Neurosci, 1995; 15: 4496-506.

Xu L, Anwyl R, Rowan MJ. Behavioural stress facilitates the induction of long-term depression in the hippocampus. Nature, 1997; 387: 497-500.

Xu L, Anwyl R, Rowan MJ. Spatial exploration induces a persistent reversal of long-term potentiation in rat hippocampus. Nature, 1998; 394: 891-4.

Yamauchi T. Neuronal Ca2+/calmodulin-dependent protein kinase II--discovery, progress in a quarter of a century, and perspective: implication for learning and memory. Biol Pharm Bull, 2005; 28: 1342-54.

Yamauchi T, Fujisawa H. Self-regulation of calmodulin-dependent protein kinase II and glycogen synthase kinase by autophosphorylation. Biochem Biophys Res Commun, 1985; 129: 213-9.

Yang CH, Huang CC, Hsu KS. Behavioral stress enhances hippocampal CA1 long-term depression through the blockade of the glutamate uptake. J Neurosci, 2005; 25: 4288-93.

Yang S-N, Tang Y-G, Zucker RS. Selective Induction of LTP and LTD by Postsynaptic [Ca2+]i Elevation. J Neurophysiol, 1999; 81: 781-7.

Yang Y, Wang XB, Frerking M, Zhou Q. Spine expansion and stabilization associated with long-term potentiation. J Neurosci, 2008; 28: 5740-51.

Yokoyama CT, Myers SJ, Fu J, Mockus SM, Scheuer T, Catterall WA. Mechanism of SNARE protein binding and regulation of Cav2 channels by phosphorylation of the synaptic protein interaction site. Mol Cell Neurosci, 2005; 28: 1-17.

Yoshimura Y, Aoi C, Yamauchi T. Investigation of protein substrates of Ca(2+)/calmodulin-dependent protein kinase II translocated to the postsynaptic density. Brain Res Mol Brain Res, 2000; 81: 118-28.

Yoshimura Y, Sogawa Y, Yamauchi T. Protein phosphatase 1 is involved in the dissociation of Ca2+/calmodulin-dependent protein kinase II from postsynaptic densities. FEBS Lett, 1999; 446: 239-42.

Yoshimura Y, Yamauchi T. Phosphorylation-dependent reversible association of Ca2+/calmodulin-dependent protein kinase II with the postsynaptic densities. J Biol Chem, 1997; 272: 26354-9.

Young JZ, Isiegas C, Abel T, Nguyen PV. Metaplasticity of the late-phase of long-term potentiation: a critical role for protein kinase A in synaptic tagging. Eur J Neurosci, 2006; 23: 1784-94.

Young JZ, Nguyen PV. Homosynaptic and Heterosynaptic Inhibition of Synaptic Tagging and Capture of Long-Term Potentiation by Previous Synaptic Activity. J. Neurosci., 2005; 25: 7221-31.

Yuste R, Majewska A, Cash SS, Denk W. Mechanisms of calcium influx into hippocampal spines: heterogeneity among spines, coincidence detection by NMDA receptors, and optical quantal analysis. J Neurosci, 1999; 19: 1976-87.

Zakharenko SS, Zablow L, Siegelbaum SA. Visualization of changes in presynaptic function during long-term synaptic plasticity. 2001; 4: 711-7.

Zhang W, Benson DL. Stages of synapse development defined by dependence on F-actin. The Journal Of Neuroscience: The Official Journal Of The Society For Neuroscience, 2001; 21: 5169-81.

Zheng F, Luo Y, Wang H. Regulation of brain-derived neurotrophic factor-mediated transcription of the immediate early gene Arc by intracellular calcium and calmodulin. J Neurosci Res, 2008.

Zhong N, Zucker RS. Roles of Ca2+, hyperpolarization and cyclic nucleotide-activated channel activation, and actin in temporal synaptic tagging. J Neurosci, 2004; 24: 4205-12.

Zhou JL, Shatskikh TN, Liu X, Holmes GL. Impaired single cell firing and long-term potentiation parallels memory impairment following recurrent seizures. Eur J Neurosci, 2007; 25: 3667-77.

Zhou Q, Homma KJ, Poo MM. Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. Neuron, 2004; 44: 749-57.

Zola-Morgan S, Squire LR, Amaral DG. Human amnesia and the medial temporal region: enduring memory impairment following a bilateral lesion limited to field CA1 of the hippocampus. J Neurosci, 1986; 6: 2950-67.